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**Tully et al.**

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(45) **Date of Patent:** **\*Aug. 11, 2009**

(54) **METHOD FOR ASSESSING THE EFFECT OF  
A DRUG ON LONG TERM MEMORY  
FORMATION**

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(\*) Notice: Subject to any disclaimer, the term of this  
patent is extended or adjusted under 35  
U.S.C. 154(b) by 95 days.

This patent is subject to a terminal dis-  
claimer.

(21) Appl. No.: **11/066,125**

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(65) **Prior Publication Data**

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**Related U.S. Application Data**

(60) Continuation of application No. 09/419,371, filed on  
Oct. 14, 1999, now Pat. No. 6,890,516, which is a  
division of application No. 08/809,917, filed as appli-  
cation No. PCT/US95/13198 on Oct. 6, 1995, now Pat.  
No. 6,689,557, which is a continuation-in-part of  
application No. 08/361,063, filed on Dec. 21, 1994,  
now Pat. No. 6,051,559, which is a continuation-in-  
part of application No. 08/319,866, filed on Oct. 7,  
1994, now Pat. No. 5,929,223.

(51) **Int. Cl.**

**A61K 49/00** (2006.01)  
**A61K 38/00** (2006.01)  
**G01N 31/00** (2006.01)

**G01N 33/53** (2006.01)

**C12Q 1/68** (2006.01)

(52) **U.S. Cl.** ..... **424/9.2**; 424/9.1; 435/6;  
435/7.1; 435/7.2; 435/7.21; 514/12; 514/44;  
436/15

(58) **Field of Classification Search** ..... None  
See application file for complete search history.

(56) **References Cited**

U.S. PATENT DOCUMENTS

6,689,577 B1 \* 2/2004 Bozsik et al. .... 435/29  
6,890,516 B1 \* 5/2005 Tully et al. .... 424/9.2  
2006/0069245 A1 \* 3/2006 Tully et al. .... 536/23.5

OTHER PUBLICATIONS

Hampton et al. *Curr. Opi. Neurobiol.* 2004. 14: 192-197.\*  
Kaang et al. *Neuron.* 1993. 10:427-435.\*  
Zhong et al. *Science* 1991. 251: 198-201.\*  
Chutae et al. *psychopharmacology.* 1981. 74: 129-31.\*  
Brindle et al. *Nature.* 1993. 364: 821-824.\*  
Foulkes et al. *Cell.* 1992. 68:411-414.\*  
Yamamoto et al., *Nature*, 1988. 334:494-498.\*  
Foulkes et al., *Cell.* 1991.64:739-749.\*  
Randt et al. *Pharmacol. Biochem. Behav.* 1982. 17: 677-80.\*  
Dash et al. *Nature.* 1990. 345: 718-721.\*  
de Groot et al. *Oncogene.* 1994. 9:463-8.\*

\* cited by examiner

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Hampton LLP

(57) **ABSTRACT**

Methods of assessing the effect of a drug on long term  
memory and for screening a pharmaceutical agent for its  
ability to modulate long term memory are disclosed.

**50 Claims, 25 Drawing Sheets**

1 ATGGACACAGCATCGTCGAGGGAGACGGCACTCGTCGSGGCATCGGGCTCCATGAC  
1 M D N S I U E E N G N S S A A S G S N D

61 GTGGTCGATGTCGTTGCCAACAGGGCGGCGGAGCGGTGGGCGGCGGGTGGAGGAGGA  
21 U U D U U A Q A A A A V G G G G G G G

121 GGAGGCGGCGGGCGGTGGTGGTAACCCCAAGCAGCAGCAACAGAACCCCAAGTACAACG  
41 G G G G G G G N P Q Q Q Q Q H P Q S T T

181 GCCGGCGGTCCACCGGGTGCAGCAGAACAGCCAGGGAGGCGGAGTGTCTCCGTGCTG  
61 A G G P T G A T N N A Q G G G U S S U L

241 ACCACCACCGCCCACTGCAACATACATACCCCATCCAGACGCTGGCGCAGCACGGACTG  
81 T T T R N C N I Q Y P I Q T L A Q H G L

301 CAGGTGAGCATTGGGGACCGGGTGGTGGTCACTGTCGAGTGTGAGGTGTTACGGA  
101 Q U S I U D P G A D C Q L S S U A C Y G  
Exon 2

361 TCCAGCCAGAGTGGCTACCAAGGATGTGCRGTCCGTGATACAGGCCAATCCCTCGGGA  
121 S D P E U A T K D U Q S U I Q A N P S G

421 GTCATACAGACAGCAGCTGGAAACCAGCAGCAGCAACAGGGCGCTGGCCCGCCACAGCG  
141 U I Q T A A G T Q Q Q Q Q A L A A A T A

481 ATGCAGAGGTGGTCTACGTGGCCARGCCGCCGACTCGACGGTCATCCACACGACGCCT  
161 N Q K U U Y U A K P P N S T U I H T T P

541 GGCAATGCAGTGCAGTGGCTARCAARTCCCTCCACCTTTCCATGTAGATCAGCCC  
181 G N A U Q U A N K I P P T F P C K I K P  
Exon 4

601 GAACCGACACGCGACCCGGAGGACAGCGACGAGGTCTGTGGACGACGATTCCACG  
201 E P N T Q H P E D S D E S L S D D D S Q

661 CACCACCGCAGCGAGCTGACGGCAGCGCGCTGACATAGATCTTCCACCGAGATCAGC  
221 H H R S E L T R R P S Y N K I F T E I S  
P-box

721 GGTCCGGACATGAGCGGCGCATCGCTTCCATGTCCGACGGCGTGGTCAATTCCACGCTG  
241 G P D N S G A S L P A S D G U L H S Q L  
Exon 6

781 GTGGGACCGGAGCGGGGGCAATGCGGCGACAGCTCCCTGATGCAATTGGATCCCACG  
261 U G T G A G G N A R N S S L M Q L D P T

841 TACTACCTGTCCATCGGATGTCTACACACCAACACAGCGGGATAGCGGAGGATCAG  
281 E Y L S N A N S Y H T H N S D I A E D Q

901 ACCCGTAAGCGCGAGATCCGGCTGCAGAGAACAGGGAGGCGGCGGTGAGTGCCGGCGC  
301 T (K)(R) E I (A) L Q (K) N (R) E A A (R) E C (R)(R)  
Basic region →

961 AAGAGAGGGAGTACATCAAGTGCCTGGAGATCGAGTGGCGGTGCTAGAGAACCAAAC  
321 (K)(K)(K) E Y I (K) C (L) E N R U A U (L) E N Q N  
Leucine zipper →

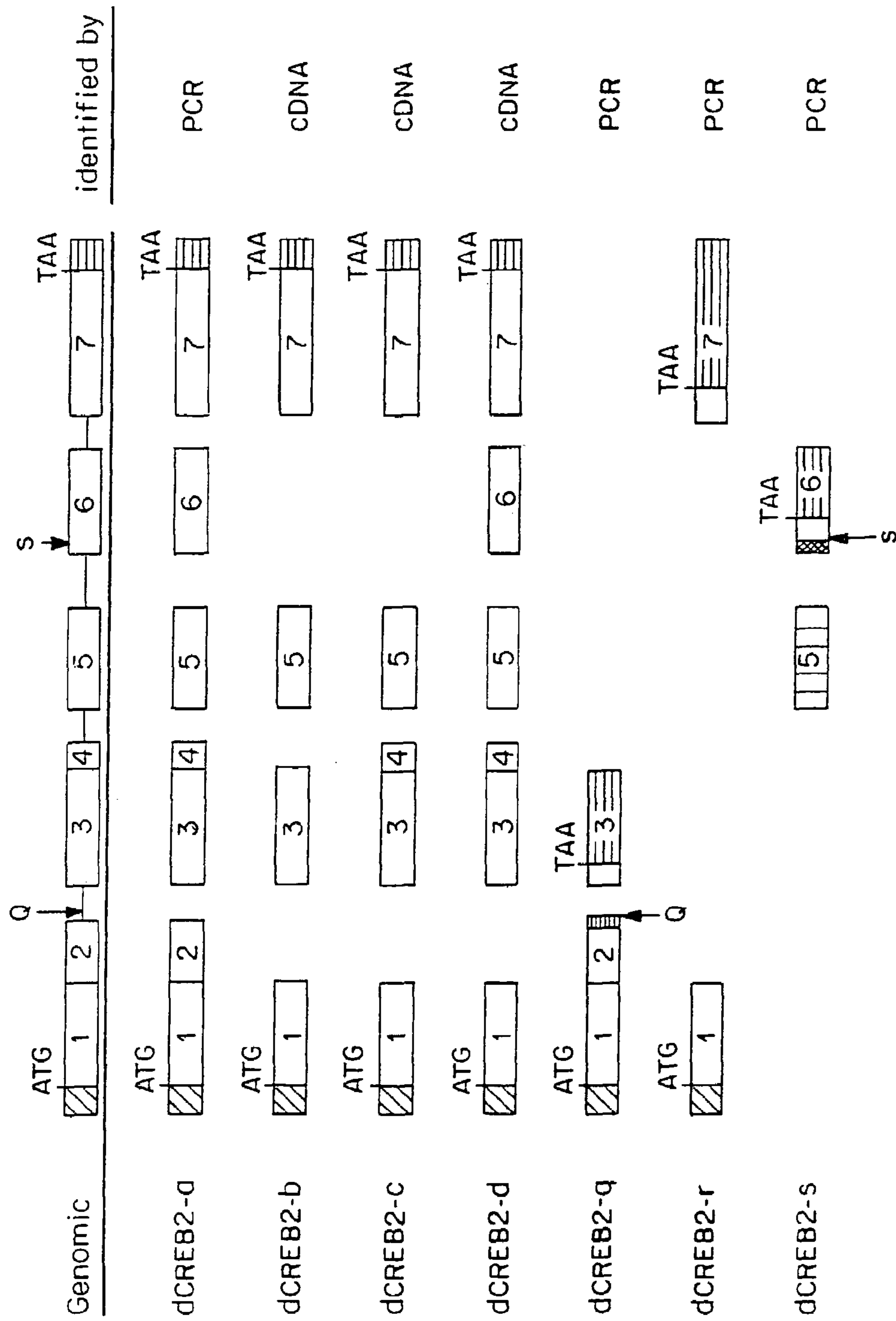
1021 AAAGCGCTCATCGAGGAGCTGAGTCTGCTCAGGAGCTCTATTGTACAGCCAGAACGAT  
341 K A (L) I E E L K S (L) K E L Y C Q T K N D

1081 TGA  
361 END

FIG. 1A

dCREB2	RKRE	RL	Q	KN	RE	A	A	R	E	C	R	R	K	K	E	Y	I	K	C	L	E	N	R	V	A	V	L	E	N	Q	N	K	A	L	I	E	E	L	K	S	L	K	E	L	Y	C			
CREB	RKRE	V	R	L	M	K	N	R	E	A	R	E	C	R	R	K	K	E	Y	V	K	C	L	E	N	R	V	A	V	L	E	N	Q	N	K	T	L	I	E	E	L	K	A	L	K	D	L	Y	C
CREM 1	RKRE	L	R	L	M	K	N	R	E	A	R	E	C	R	R	K	K	E	Y	V	K	C	L	E	N	R	V	A	V	L	E	N	Q	N	K	T	L	I	E	E	L	K	A	L	K	D	L	Y	C
ATF-1	LKRE	I	R	L	M	K	N	R	E	A	R	E	C	R	R	K	K	E	Y	V	K	C	L	E	N	R	V	A	V	L	E	N	Q	N	K	T	L	I	E	E	L	K	T	L	K	D	L	Y	S

FIG. 1B



  = coding sequences.   = 5' untranslated sequences.   = 3' untranslated sequences.   = intron  
  = coding sequence excluded by alternative splicing.   = coding sequence excluded by alternative splicing.  
  = portion of exon included in spliced transcript is unknown.

FIG. 2

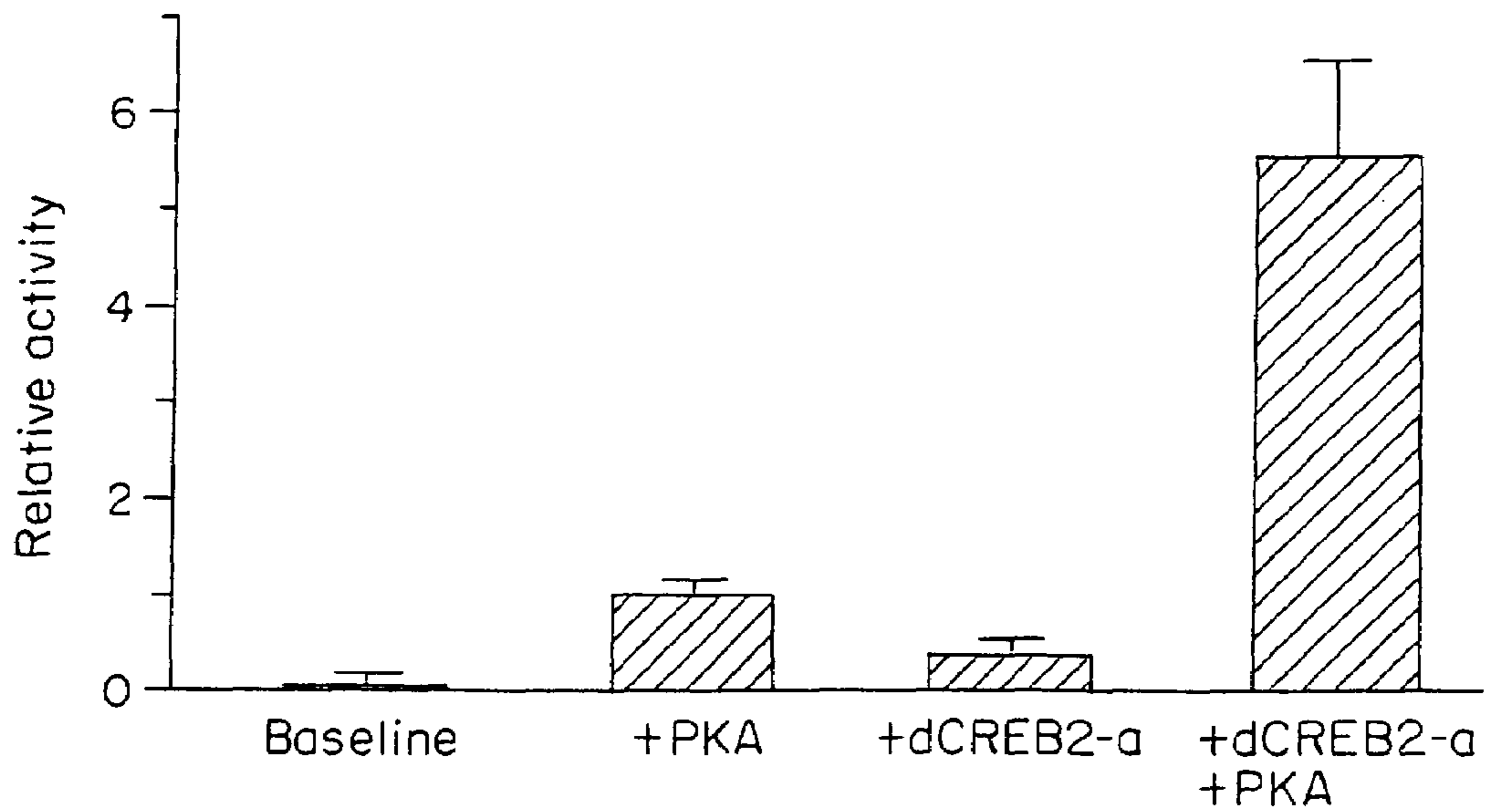


FIG. 3

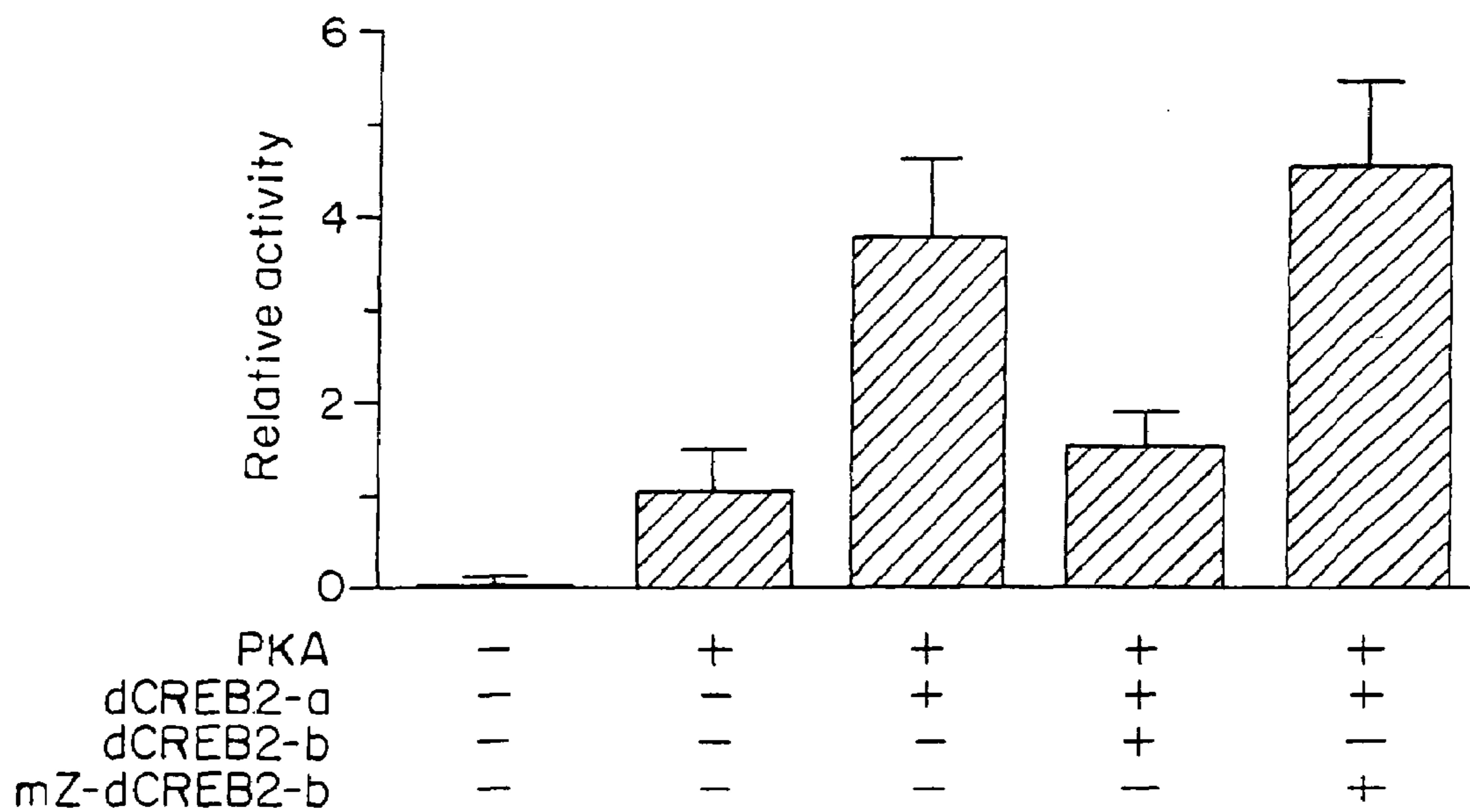


FIG. 4

1 ATGTTACTCGGAGAAATATGTTTTCTACTTTCCATCGTTAGATGCTGCTACCGCTACA  
 1 M L L G E N N F S T F T S L D A A T A T  
  
 61 ACCAACCCGGTGAATTCTTAATGAATGAATCTCCRAGGCAAGAGCCGGTGACTTARTG  
 21 T N T G E F L N N E S **P** R Q E A G D L N  
  
 121 TTGGATAGTCTGGATTTCAACATTATGGGCGAAACCTGGCAGATGATTTCCAGACCTCG  
 41 L D S L D F N I N G E N L A D D F Q T S  
  
 181 GCTTCACCAGCTTCGGAGGACRAGATGACTCCTTTCGTTGTTGATACCAATGTTTTTGA  
 61 A S P A S E D K N T **P** F U U D T N U F E  
  
 241 TCCGTCTTCRAGAACCCGAGATACCCTTCTAGGGATATCGACAATGTTGGTATTGTT  
 81 S U F K N T E D T L L G D I D N U G I U  
  
 301 GACACGGGAGTTGAGGAGATGTTCCGATTTGGTTGACTCGGAATCAATAACGGCACTCCT  
 101 D T E L K E N F D L U D S E I N N G T **P**  
  
 361 ATCAGCAGGAGAAAGGATGATTTGGATTTACTTCAAGATCCCAGTCCACCTCAGCT  
 121 I K Q E E K D D L E F T S A S Q S T S A  
  
 421 CTCTTGTCGTCGAAATCGACTTCTGCTTCTCCRAGCTGATGCTGCCGCTGCATGTGCAGT  
 141 L L S S K S T S A S **P** A D A R A R A C R S  
  
 481 CCTTCGTCATCGTCTTGTAAGATCCTATTCTTCTGCTCAGCTAGAAACTACGGGTTCCG  
 161 P S S S S C K R S Y S S A Q L E T T G S  
  
 541 GATGCTCCRAGRAGATAGCTGGGCTGCACCCCTTCACTAGAAACAGAGAAACAT  
 181 D A P K K D K L G C T P Y T R K Q R N N  
  
 601 CCATTACCTCCGGTCATTCCRAGGGTCRGGATGTTGCTTCTATGAAAAGGGCAAGAAAC  
 201 P L P P U I P K G Q D U A S N **K** **R** **R** **R** N  
Basic region →  
 661 ACTGAGGCCGCAAGAGATCAAGAGCCAGAAAATGGAAAGAAATGTCCCAACTTGAAGAA  
 221 T E A A **R** **R** S **R** A **R** **K** M E **R** N S Q **L** E E  
Leucine  
 721 AAGTGTCAAGCTTGTGAAAGGAAACGACGACTTGAAGCTCAAGTTCAGCTTTGAAG  
 241 K C Q S **L** L K E N D D **L** K A Q U Q A **L** K  
zipper →  
 781 AAATTACTTGGACAACAA  
 261 K L L G Q Q  
|||||

FIG. 5

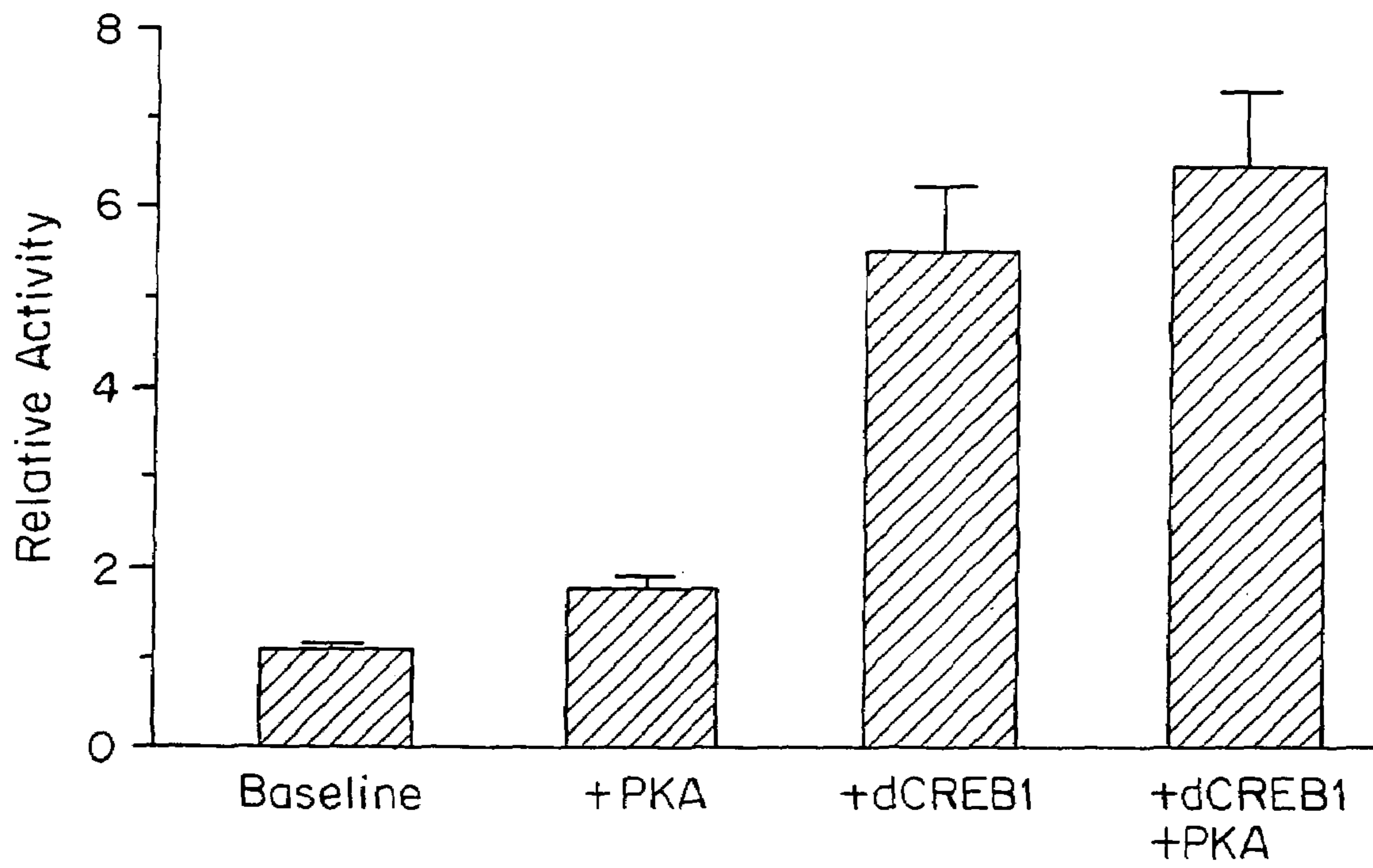


FIG. 6

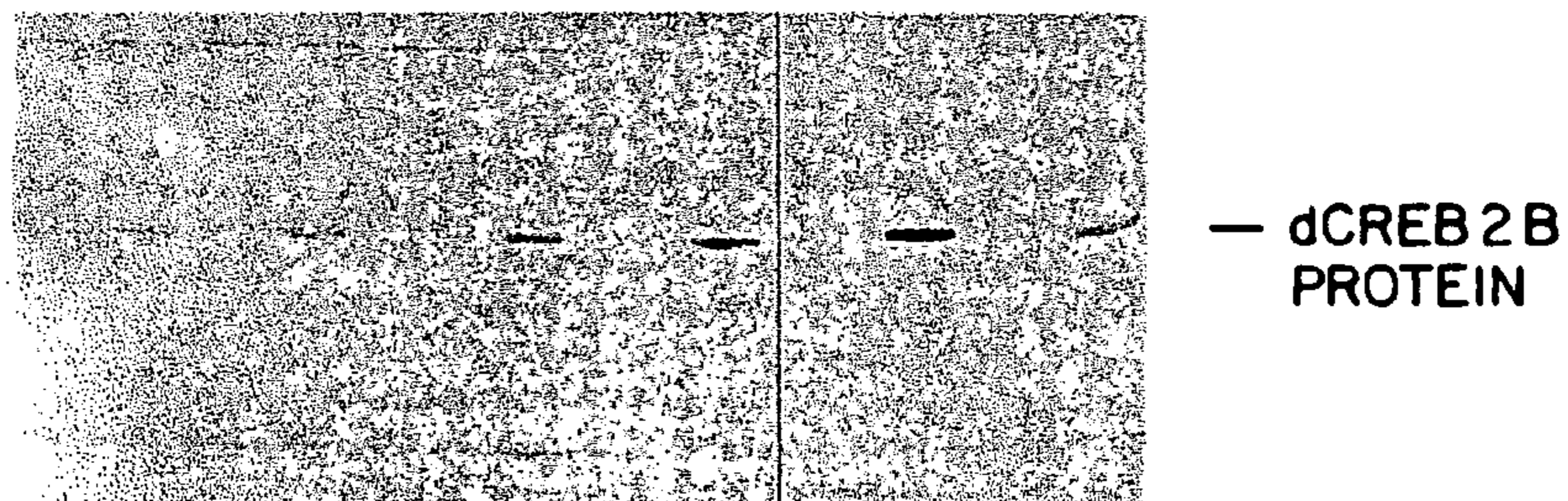
LANE #	1	2	3	4	5	6
HEAT SHOCK	-	-	+	+	+3	+3
FLIES	wt	CREB	wt	CREB	wt	CREB

FIG.7A



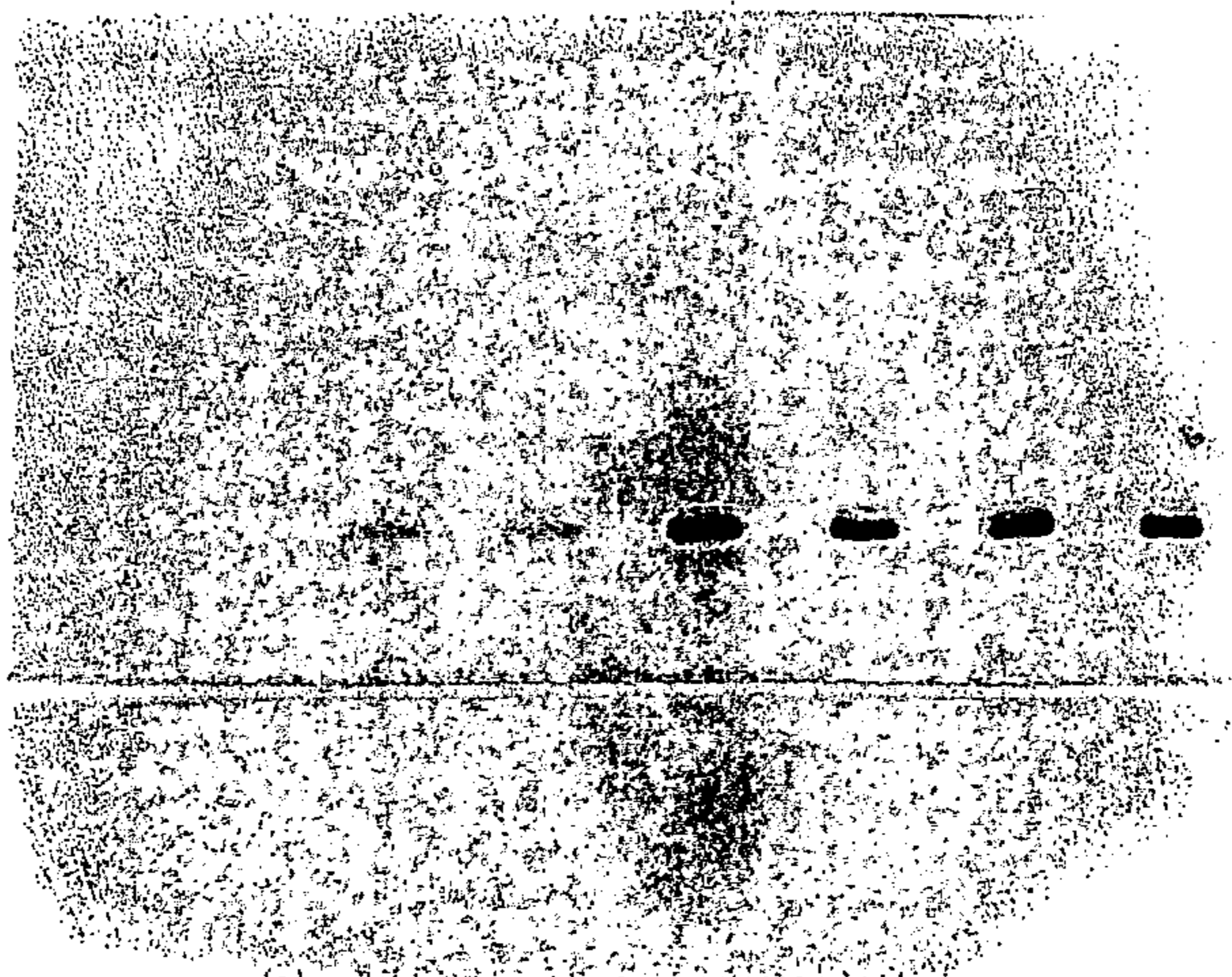
LANE #	1	2	3	4	5	6	7	8	9	10	11	12
HEAT SHOCK	-	-	+	+	+1	+1	+3	+3	+9	+9	+24	+24
FLIES	wt	CREB	wt	CREB	wt	CREB	wt	CREB	wt	CREB	wt	CREB

FIG.7B



lane	1	2	3	4	5	6	7	8
blocker	wt	m	wt	m	wt	m	wt	m
hs	-	-	+	+	+3	+3	+6	+6

FIG.7C





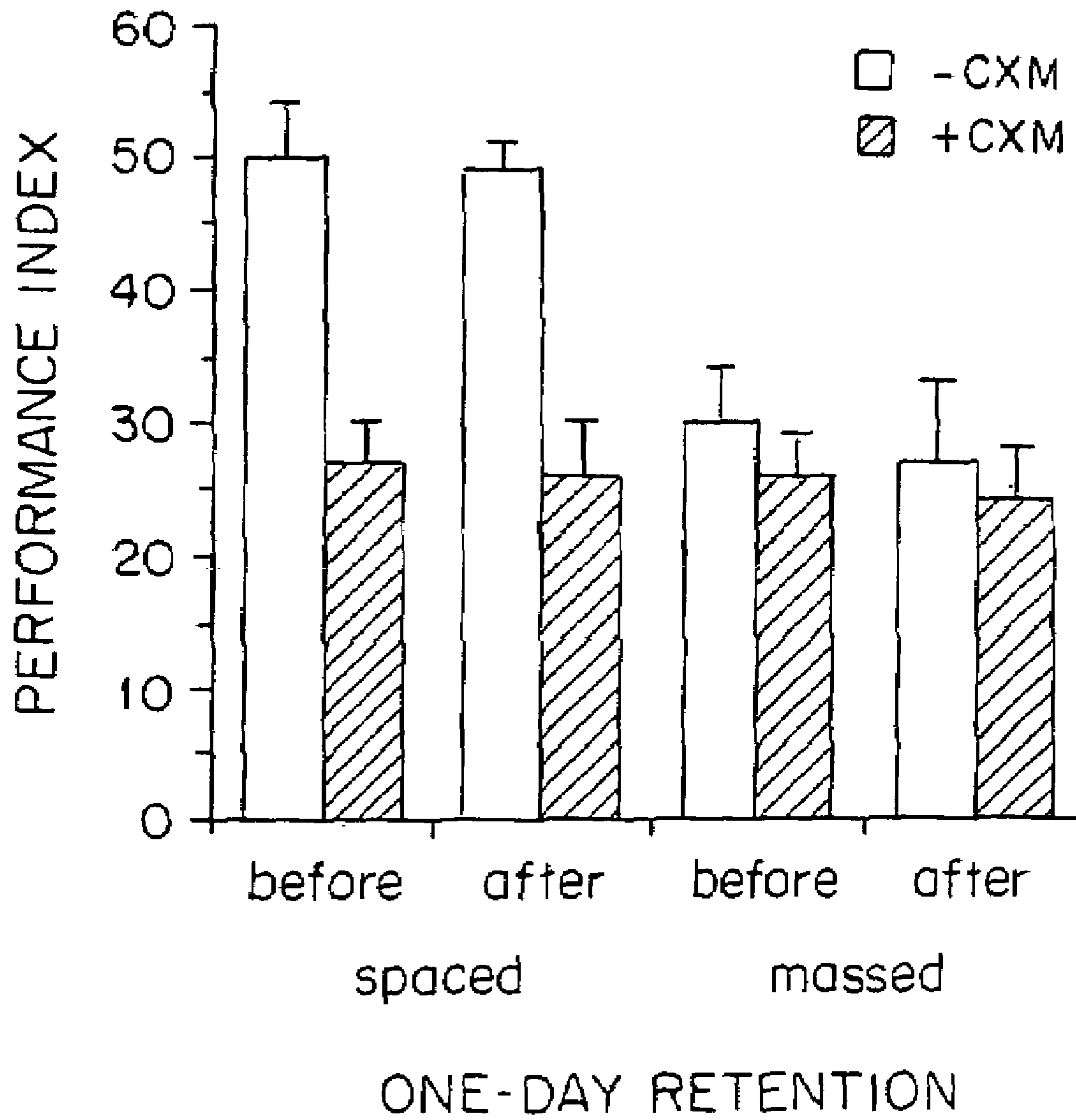


FIG. 8

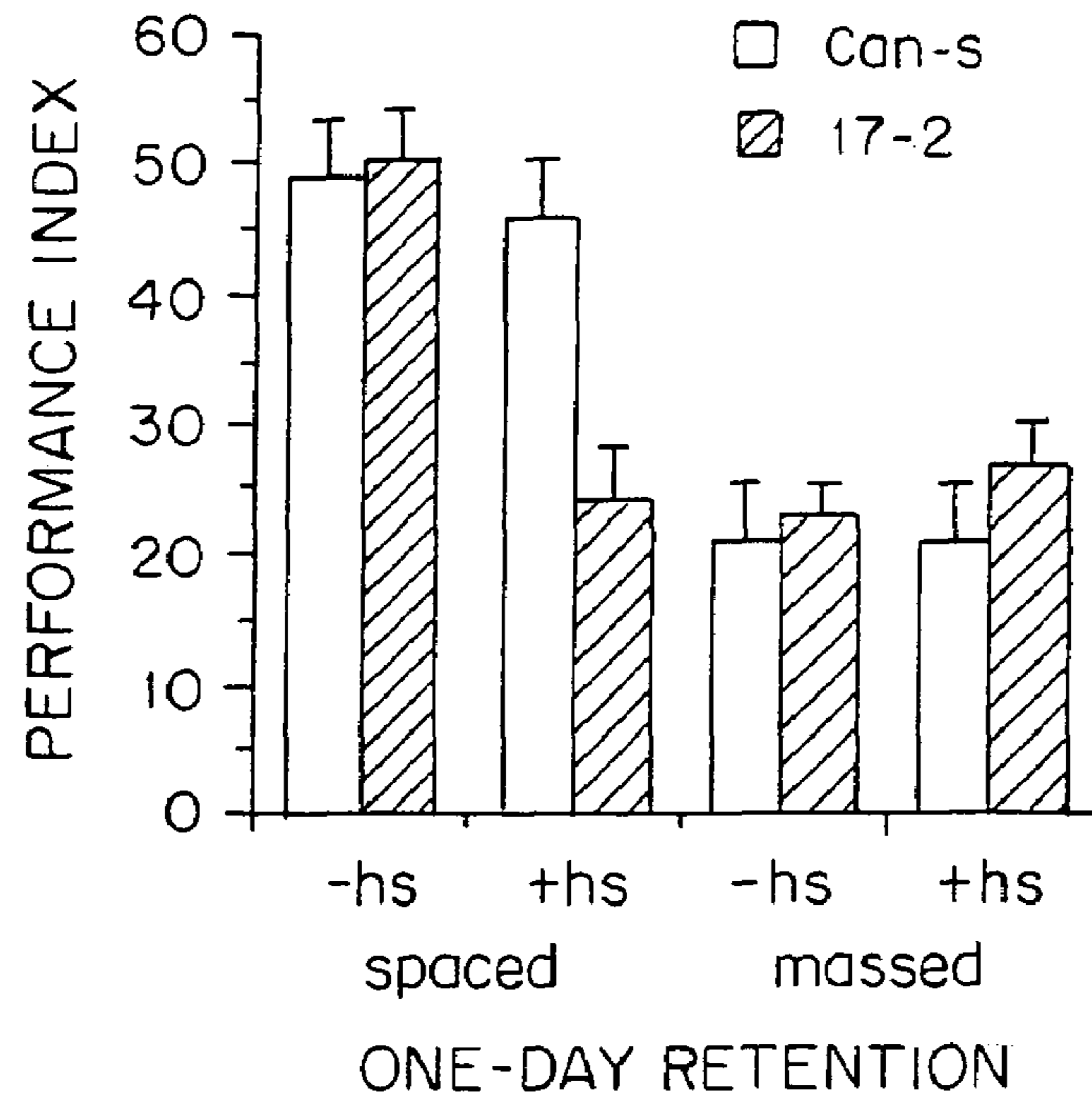


FIG. 9A

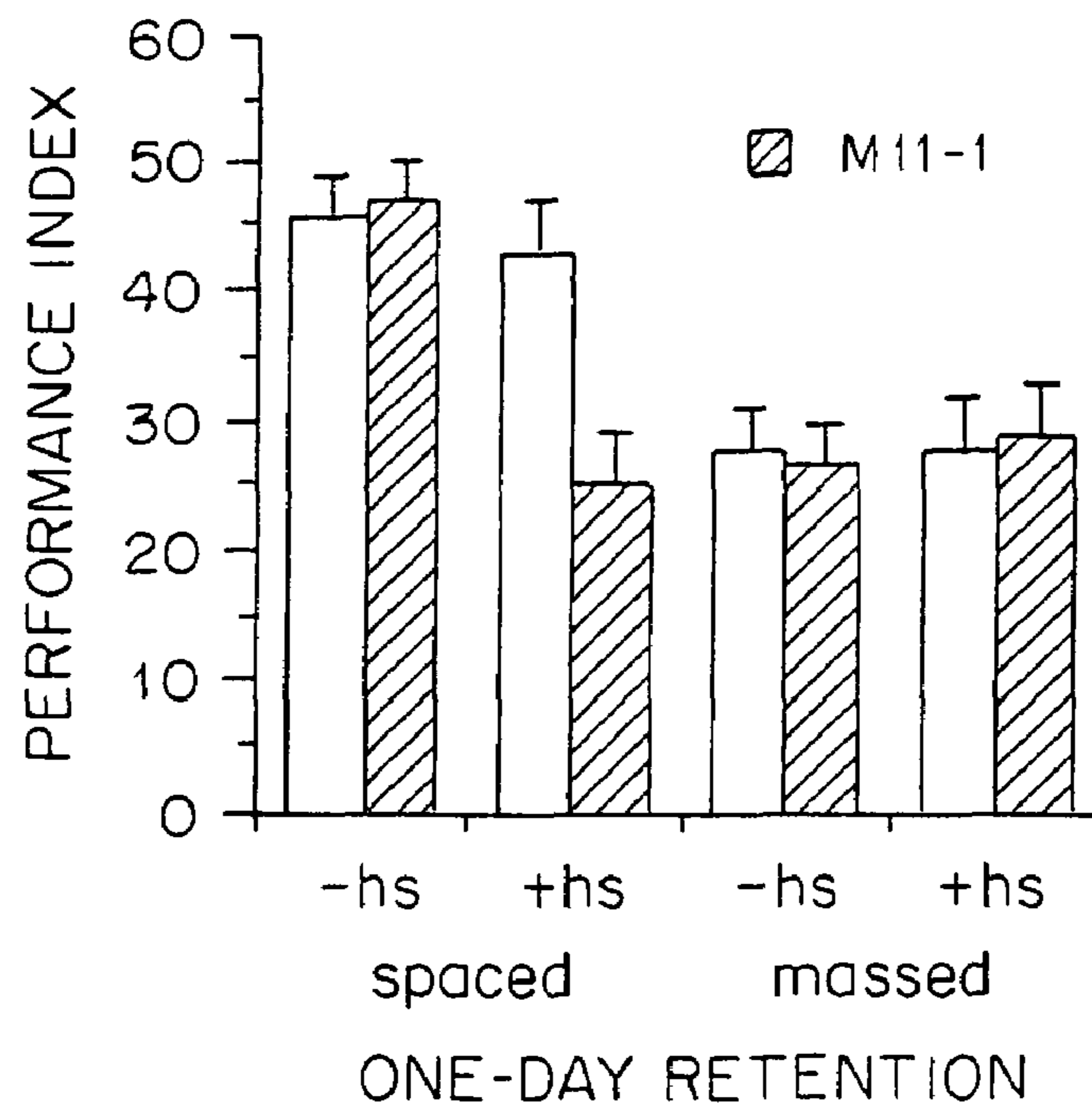


FIG. 9B

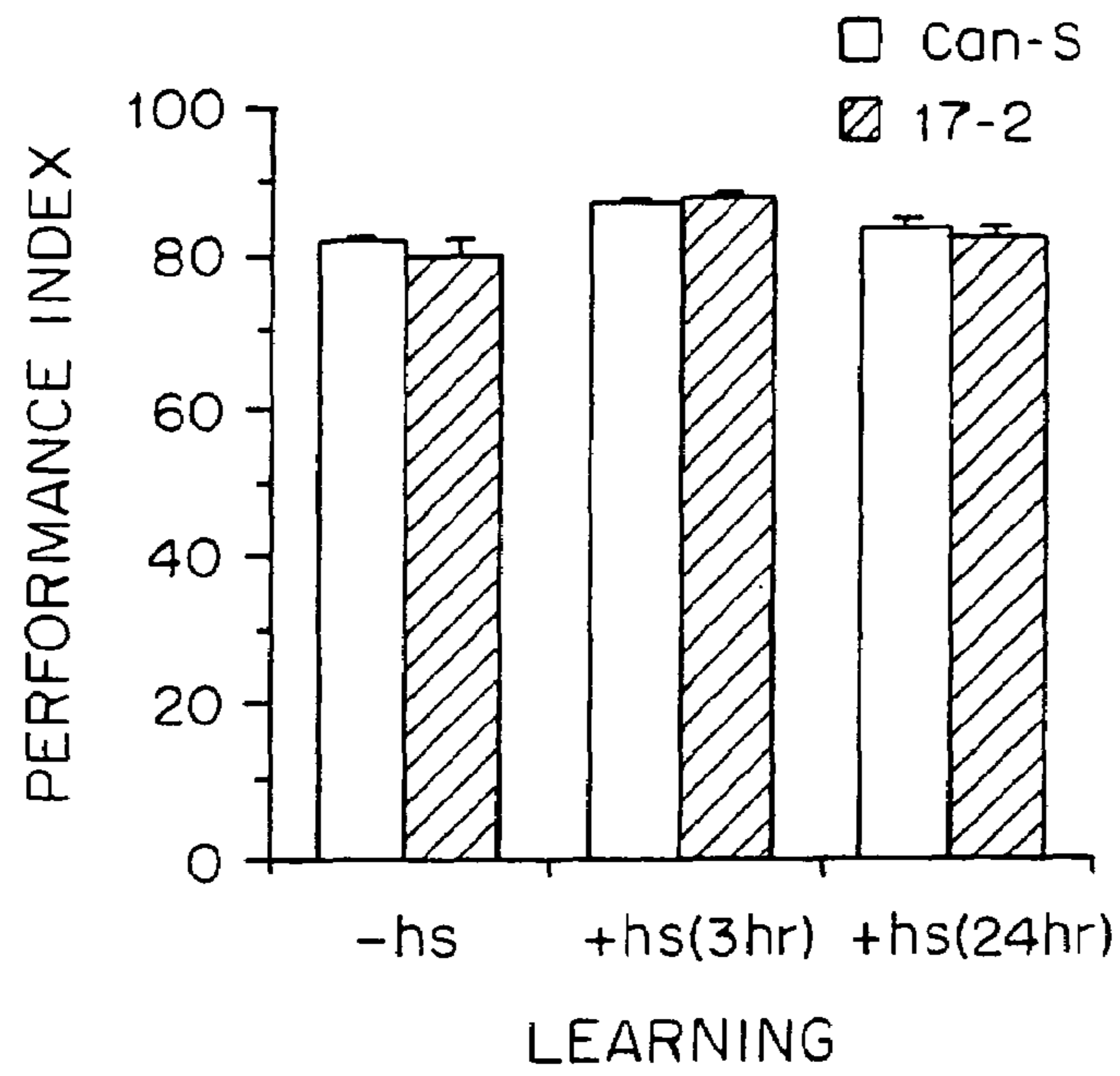


FIG. 9C

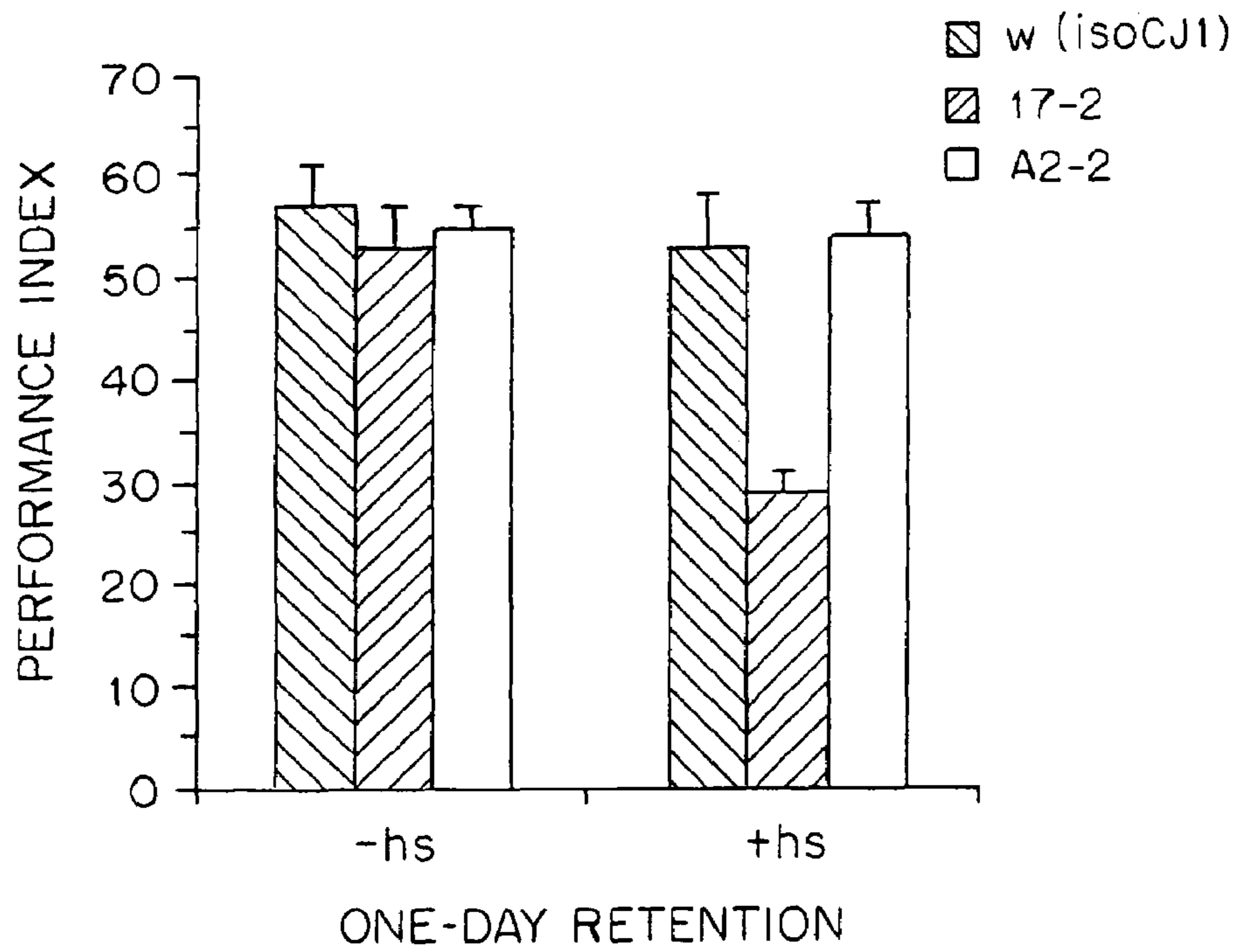


FIG. 10

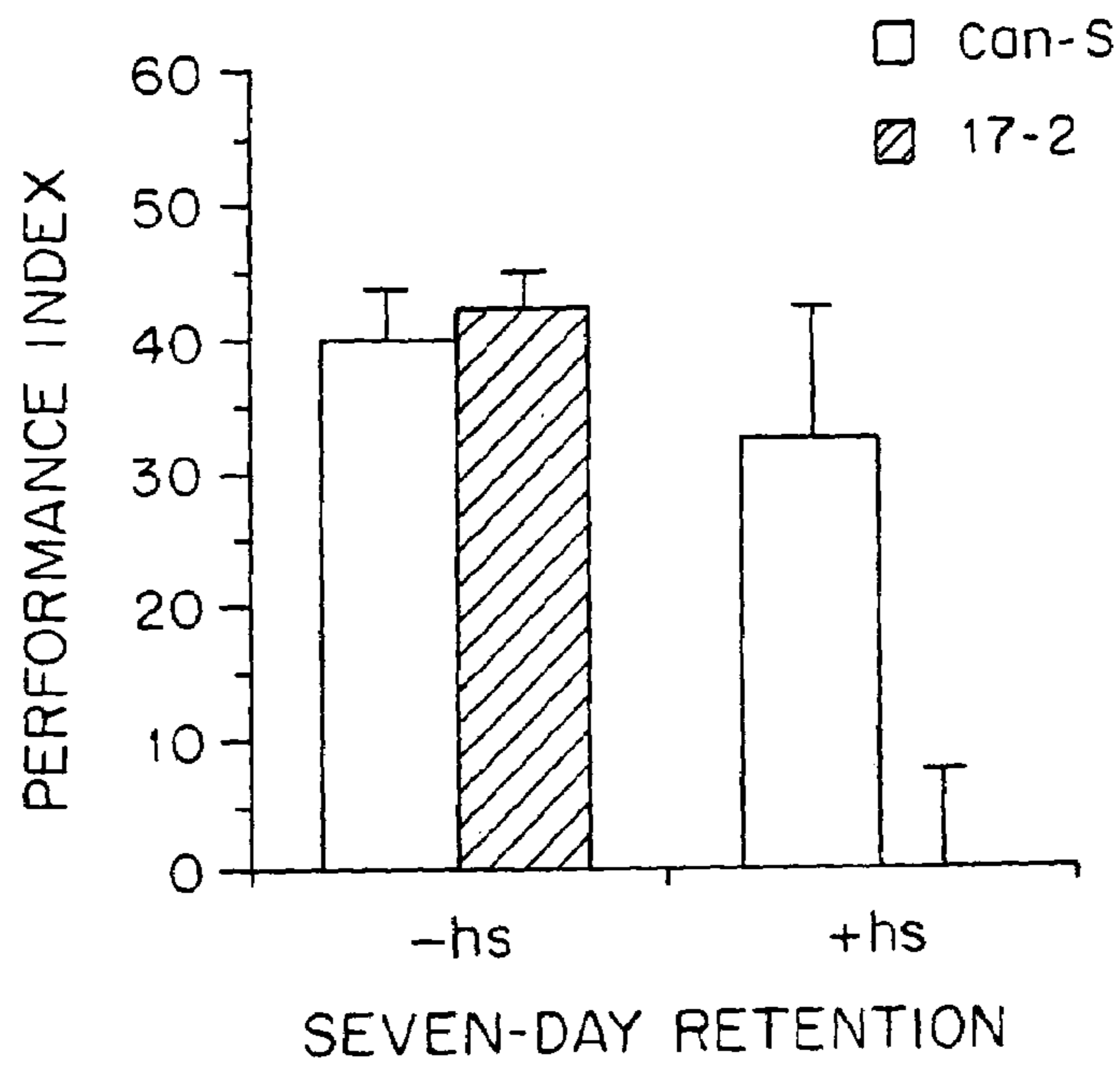


FIG. 11

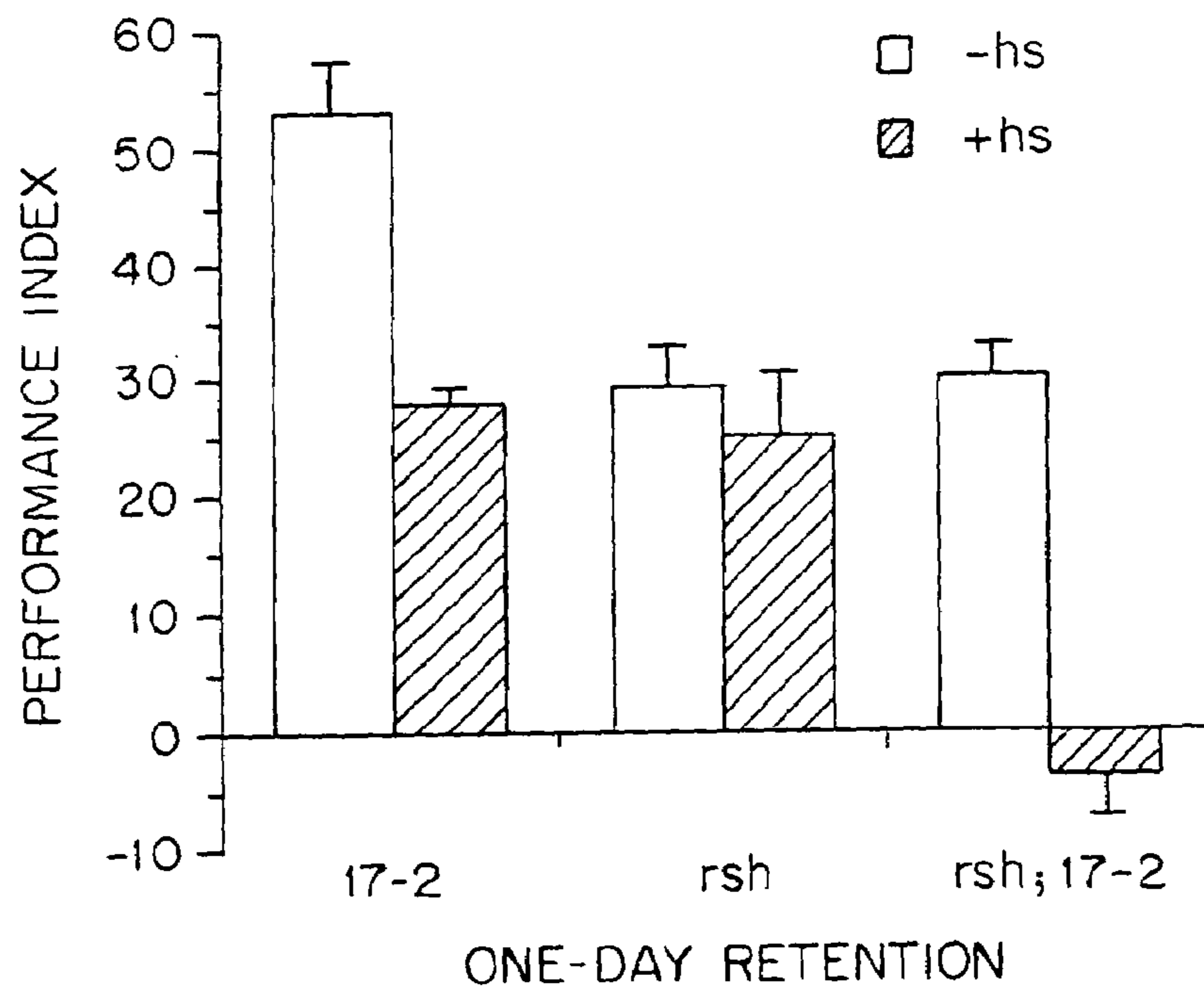


FIG. 12

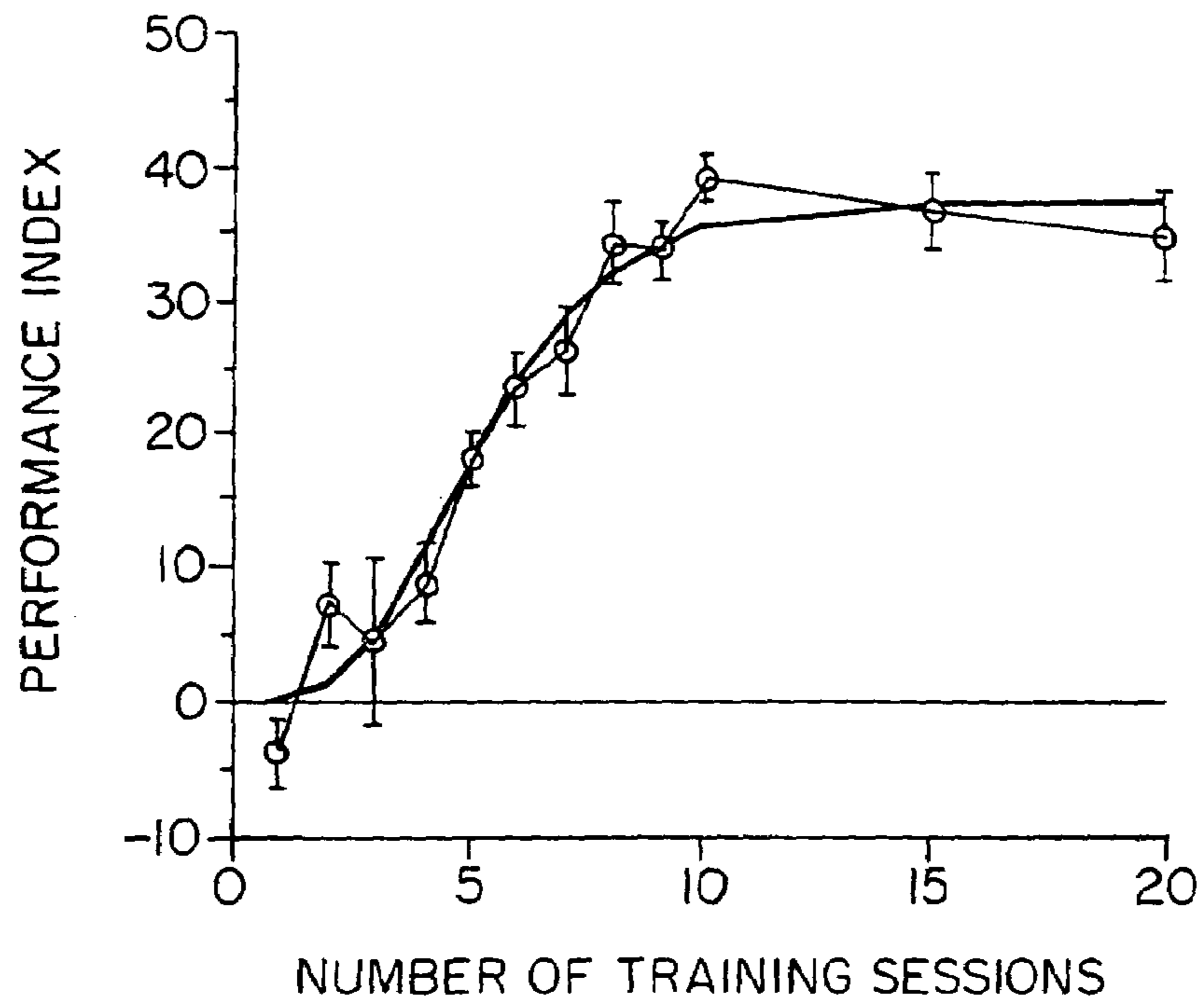


FIG. 13A

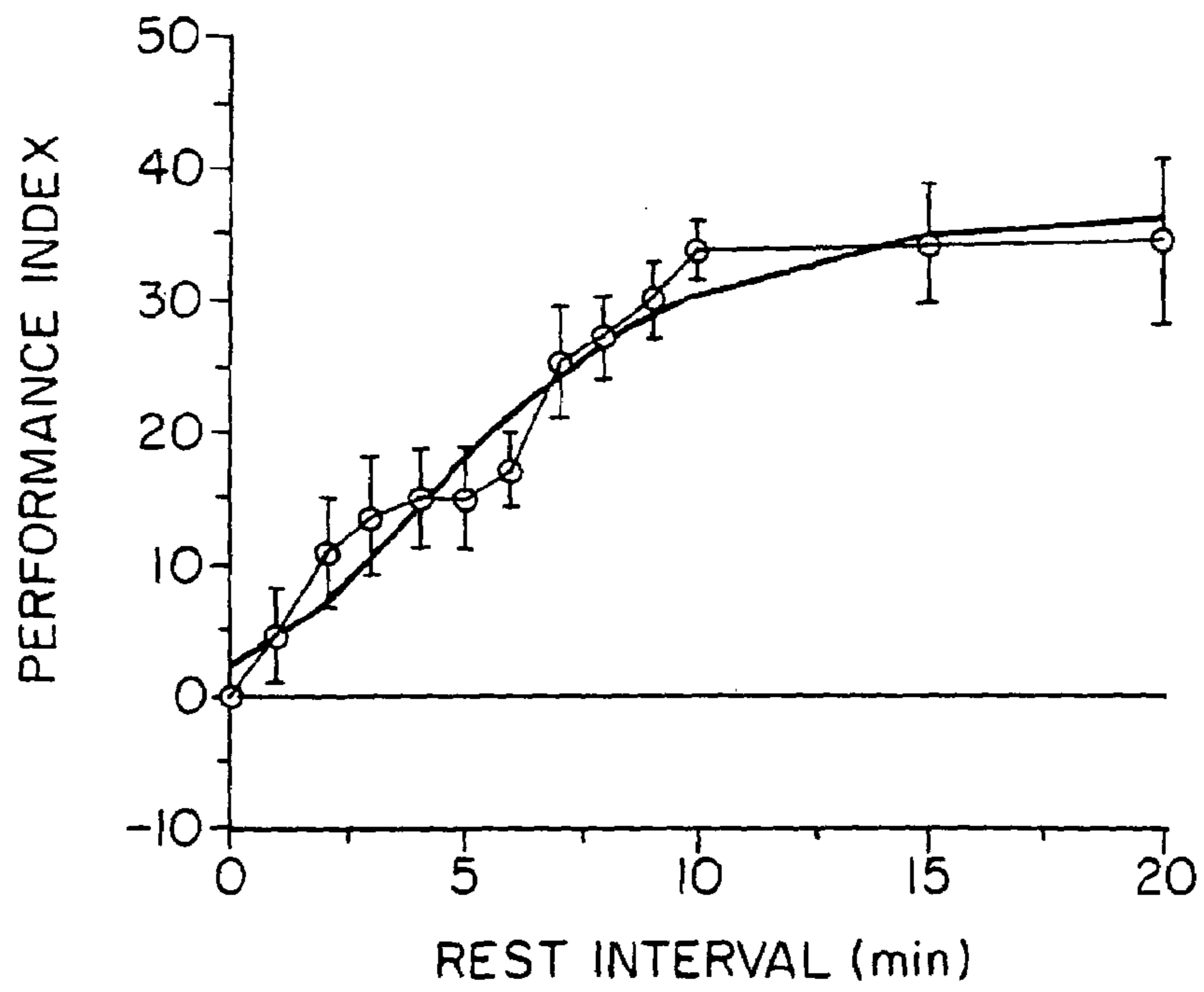


FIG. 13B

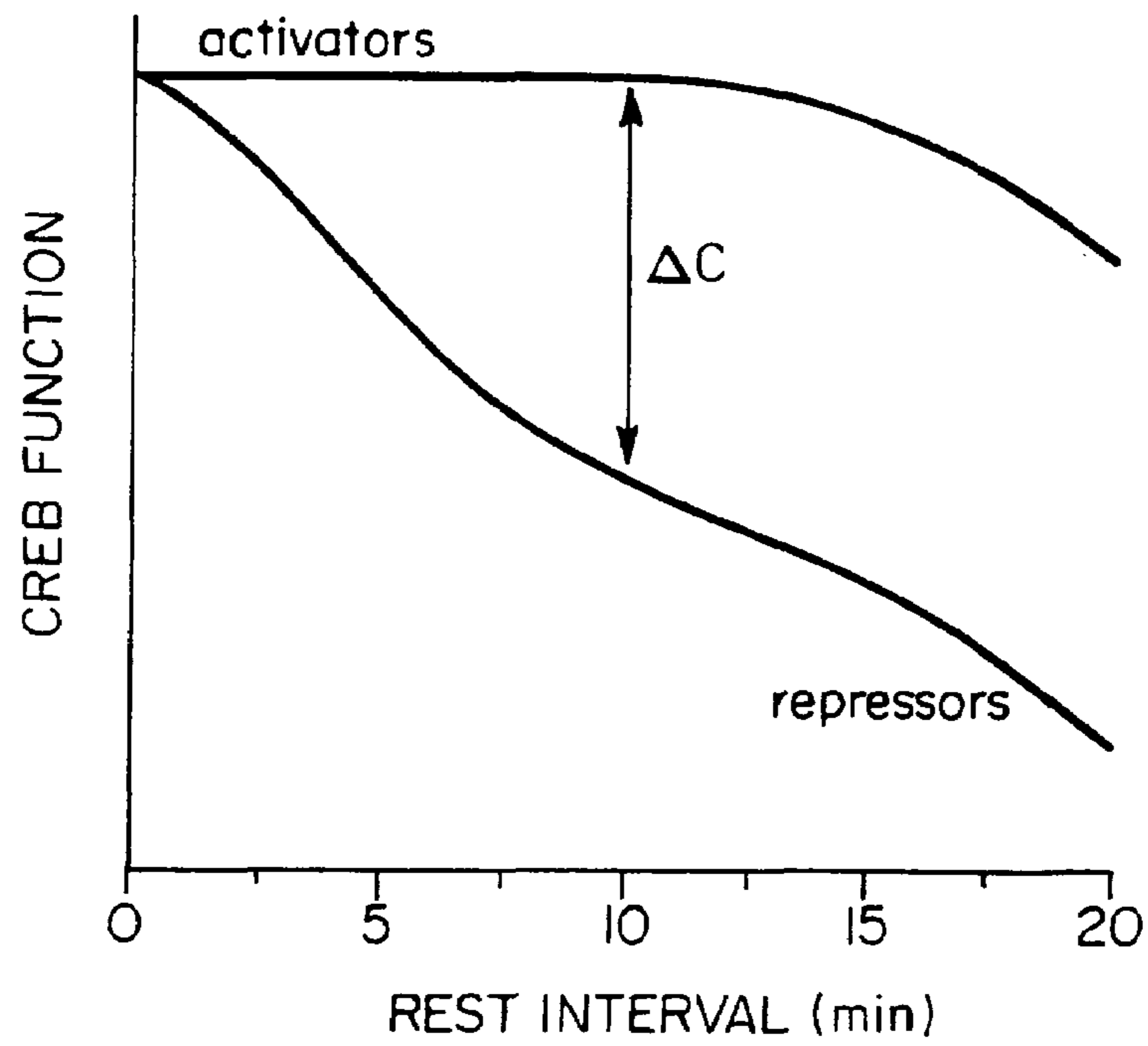


FIG. 14

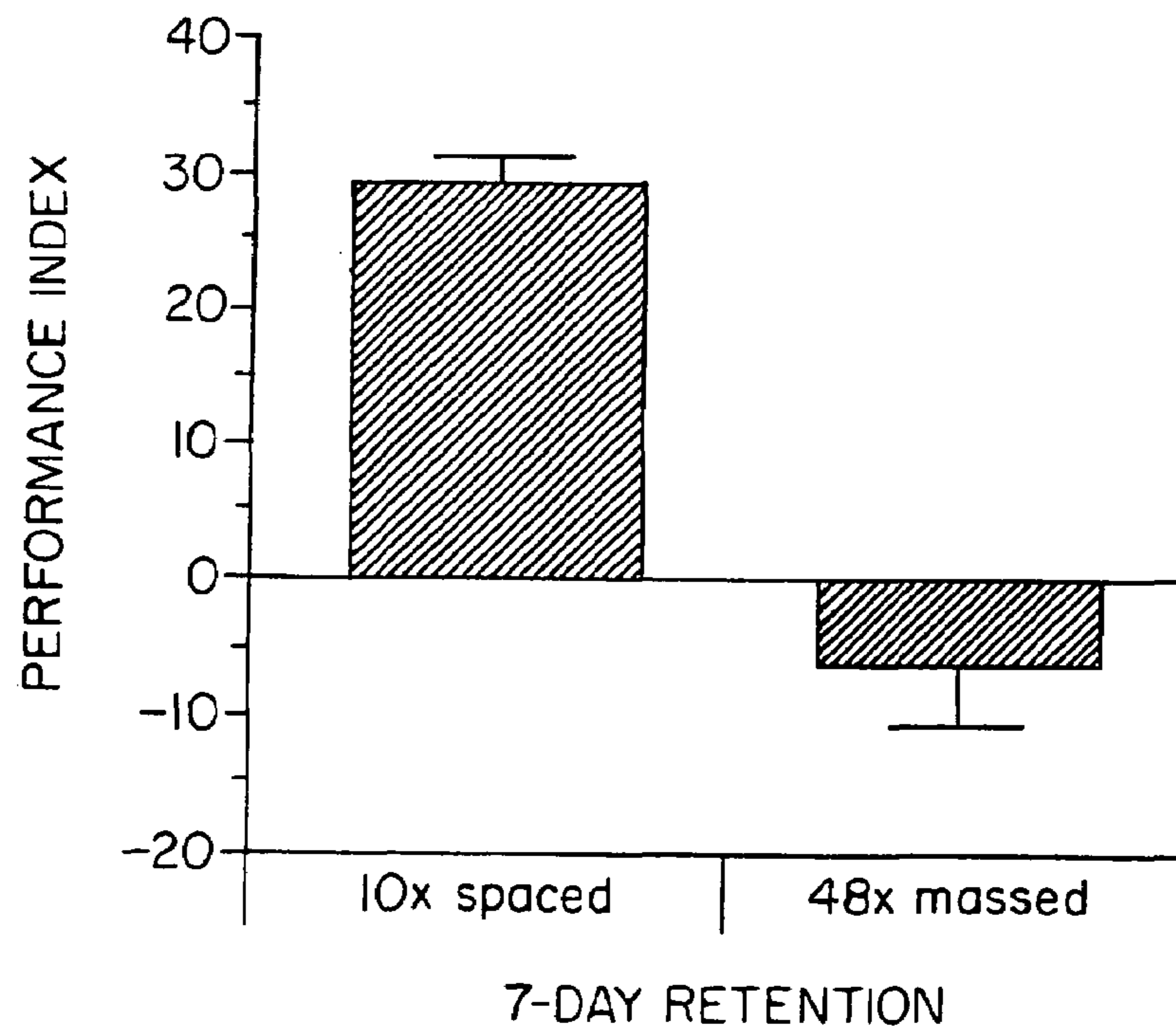


FIG. 15A

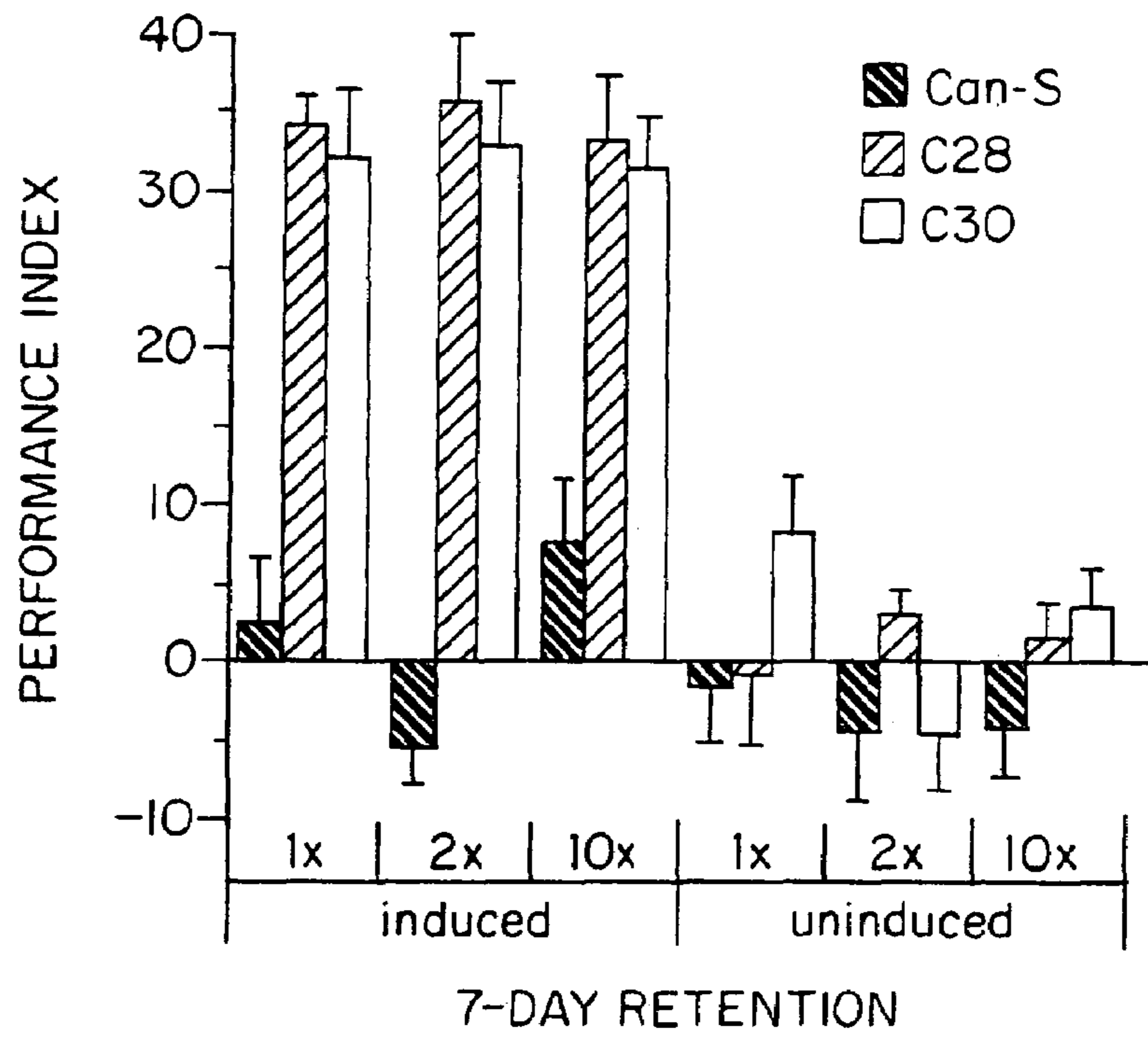


FIG. 15B

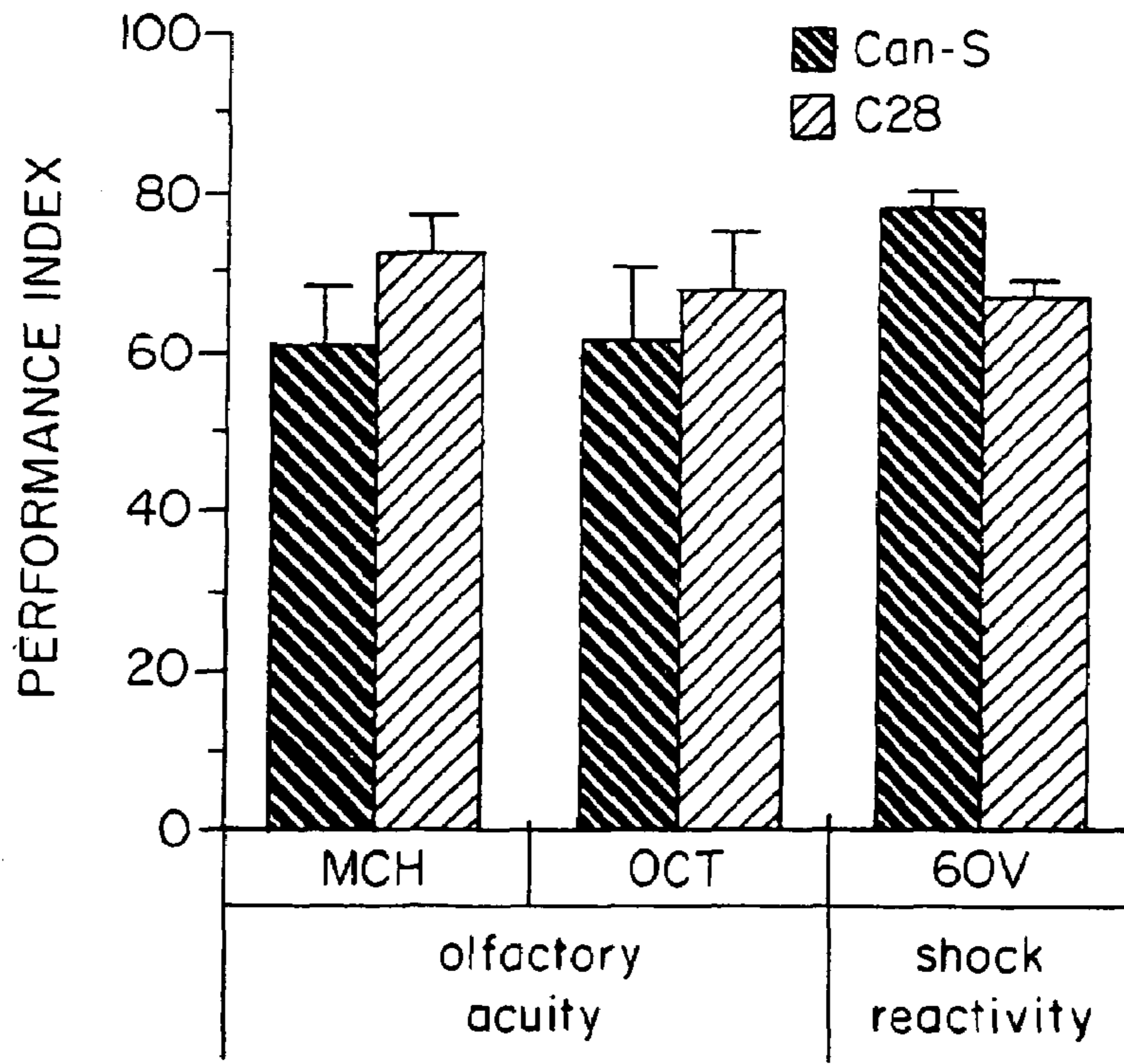


FIG. 15C

DNOS	MSQ-----H	FTSIFENLRF	VTIKRATNAQ	QQQQQQQQQQ	L-----	35
BENOS	MGN-----	-----L	--KSVG----	--QE--PGPP	-----	14
RNNOS	MEENTFGVQQ	IQPNVISVRL	FKRKVGGLGF	LVKERVSKPP	VIISDLIRGG	50
MMNOS	MAC-----	-----PWKFL	FKVKSYS----	--QSDLKEEK	-----	22
DNOS	QQQQQQQLQQQ	-----	-----KAQTQ	QQNSRKIKTQ	ATPTLNGNGL	70
BENOS	----CGL---	-----	-----G	L----GLG--	----LGLCGK	28
RNNOS	AAEQSGLIQA	GDIILAVNDR	PLVDLSYDSA	LEVLRGIASE	THVVLILRGP	100
MMNOS	----DI---	-----	-----N	N----NVK--	----KTPCAV	35
DNOS	LS-GNPNGGG	GDSSPSHEVD	HPGGAQGAQ-	-----	-----AAG	101
BENOS	QG-----	-PASPAPEPS	RAPAPATP--	-----	-----	47
RNNOS	EGFTTHLETT	FTGDGTPKTI	RVTQPLGPPT	KAVDLSHQPS	ASKDQSLAVD	150
MMNOS	LS-----	PTIQDDPKSH	QNGSPQLL--	-----	-----	55
DNOS	GLPSLSGTPL	RHH-----	---KRASIST	ASPPIRERRG	-----	131
BENOS	-----HAPD	HS-----	-----	-PAPNS----	-----	58
RNNOS	RVTGLGNGPQ	HAQGHGQAG	SVSQANGVAI	DPTMKSTKAN	LQDIGEHDEL	200
MMNOS	-----TGTA	QN-----	-----	--VPESL---	-----	66
DNOS	--TNTSIVVE	LDGSGSGSGS	GG-----	GGVGVGQAG	CPPSGSCTAS	171
BENOS	----PTLT-	-----	-----	-----R---	-----PPEG	67
RNNOS	LKEIEPVLSI	LNSGSKATNR	GGPAKAEMKD	TGIQVDRDL	GKSHKAPPLG	250
MMNOS	----DKLHV	-----	-----	-----	-----TSTR	75
DNOS	GKSSRELSPS	PKNQQQPRKM	SQDYRSR---	-AGSEMHLDD	EGRSLLMRKP	217
BENOS	-----	-----	-----	-----	-----PKF	70
RNNOS	GDNDRVFNDL	WGKDNVPVIL	NNPYSEKEQS	PTSGKQSPTK	NGSPSRCPRF	300
MMNOS	-----	-----	-----	-----	-----PQY	78
DNOS	MRLKNIEGRP	EYDRTLHCKG	REILSCSKAT	CTSSIMN---	-IGNAAVEAR	263
BENOS	PRVKNWELGS	ITYDTLCAQS	QQDGPCTPRR	CLGSLVLPK	LQTRPSGPP	120
RNNOS	LKVKNWETDV	VLDTLHLKS	TLETGCTEHI	CMGSIMLPSQ	-HTRKPEDVR	349
MMNOS	VRIKNWGSGE	ILHDTLHKA	TSDFTCKSKS	CLGSIMNPKS	LTRGPRDKPT	128
DNOS	KSDLILEHAK	DFLEQYFTSI	KRTSCTAHET	RWKQVRQSIE	TTGHYQLTET	313
BENOS	PAEQLLSQAR	DFINQYYSI	KRSGSQAHEE	RLQEVEAEVA	STGTIHLRES	170
RNNOS	TKDQLFPLAK	EFLDQYYSI	KRFGSKAHMD	RLEEVNKEIE	STSTYQLKDT	399
MMNOS	PLEELLPHAI	EFINQYYSF	KEAKIEEHLA	RLEAVTKEIE	TTGTYQLTLD	178
-----Heme-----						
DNOS	ELIYGAKLAW	RNSSRCIGRI	QWSKLQVFDC	RYVTTTSGMF	EAICNHIKYA	363
BENOS	ELVFGAKQAW	RNAPRCVGRI	QWGKLQVFDA	RDCSSAQEMF	TYICNHIKYA	220
RNNOS	ELIYGAKHAW	RNASRCVGRI	QWSKLQVFDA	RDCTTAHGMF	NYICNHVKYA	449
MMNOS	ELIFATKMAW	RNAPRCIGRI	QWSNLQVFDA	RNCSTAQEMF	QHICRHILYA	228
DNOS	TNKGNLRSAL	TIFPQRTDAX	HDIYRIWNNQL	ISYAGYKQAD	GKIIIGDPMNV	413
BENOS	TNRGNLRSAL	TVFPQRAPGR	GDFRIWNSQL	VRYAGYRQOD	GSVRGDPANV	270
RNNOS	TNKGNLRSAL	TIFPQRTDAX	HDFRWNNSQL	IRYAGYKQPD	GSTLGDPANV	499
MMNOS	TNNGNIRSAI	TVFPQRSDBG	HDFRLWNSQL	IRYAGYQMPD	GTIRGDAATL	278
DNOS	EFTEVCTKLG	WKSXGSEWDI	LPLVVSANGH	DPDYFDYPPE	LILEVPLTHP	463
BENOS	EITELCIQHG	WTPGNGRFDV	LPLLLQAPDE	APELFLVLPPE	LVLEVPLGAP	320
RNNOS	QFTEICIQQG	WKAPRGRFDV	LPLLLQANGN	DPELFIQIPPE	LVLEVPIRHP	549
MMNOS	EFTQLCIDLG	WKPRYGRFDV	LPLVLQADGQ	DPEVFEIPPD	LVLEVMEHP	328
DNOS	KFEWFSDLGL	RWYALPAVSS	MLFDVGGIQF	TATTFSGWYM	STEIGSRNLC	513
BENOS	HTGVVRGPGL	RWYALPAVSN	MLEIGGLEF	SAAPFSGWYM	STEIGTRNLC	370
RNNOS	KFDWFKDLGL	KWYGLPAVSN	MLEIGGLEF	SACPFSGWYM	GTEIGVRDYC	599
MMNOS	KYEFWQELGL	KWYALPAVAN	MLEVGGLEF	PACPFNGWYM	GTEIGVRDFC	378

FIG. 16A



DNOS	DTNRRNMLET	VALKMQLDTR	TPTSLWKDKA	VVEMNIAVLH	SYQSRNVTIV	563
BENOS	DPHRYNILED	VAVCMDLDTR	TTSSLWKDKA	AVEINLAVLH	SFQLAKVTIV	420
RNNOS	DNSRYNILEE	VAKKMDLDMR	KTSSLWKDQA	LVEINIAVLY	SFQSDKVTIV	649
MMNOS	DTQRYNILEE	VGRRMGLETH	TLASLWKDRA	VTEINVAVLH	SFQKQNVTIM	428
DNOS	DHHTASESFM	KHFENESKLR	NGCPADWIWI	VPPLSGSITP	VFHQEMALYY	613
BENOS	DHHAATVSFM	KHLDNEQKAR	GGCPADWAWI	VPPIYGSLLP	VFHQEMVNYI	470
RNNOS	DHHSATESFI	KHMENEYRCR	GGCPADVWVI	VPPMSGITP	VFHQEMLNYR	699
MMNOS	DHHTASESFM	KHMONEYRAR	GGCPADWIWL	VPPVSGSITP	VFHQEMLNYV	478
-----CaM-----						
DNOS	LKPSFEYQDP	AWRTHVWKKG	RGESKGGKPR	RKFNFKQIAR	AVKFTSKLFG	663
BENOS	LSPAFRYQPD	PWKG---SAT	KGAG---ITR	KK-TFKEVAN	AVKISASLMG	513
RNNOS	LTPSFEYQPD	PWNTHVWKG	NGTP---TKR	RAIGFKKLAE	AVKFSAKLMG	746
MMNOS	LSPFYQQIE	PWKTHIWQNE	KLRP----RR	REIRFRVLVK	VVFFASMLMR	524
DNOS	RALSKRIKAT	VLATETGKS	EQYAKQLCEL	LGHAFNAQIY	CMSDYDISSI	713
BENOS	TLMARVKAT	ILYASETGRA	QSYAQQLGRL	FRKAFDPRVL	CMDEYDVVSL	563
RNNOS	QAMARVKAT	ILYATETGKS	QAYAKTLCEI	FKHAFDAKAM	SMEEYDIVHL	796
MMNOS	KVMASVRAT	VLFATETGKS	EALARDLATL	FSYAFNTKVV	CMDQYKASTL	574
DNOS	EHEALLIVVA	STFGNGDPPE	NGELFSQELY	AMRVQESSEH	GLQDSSIGSS	763
BENOS	EHEALVLVVT	STFGNGDPPE	NGESFAAALM	EMSGPYNS--	---SPRPEQH	608
RNNOS	EHEALVLVVT	STFGNGDPPE	NGEKFGCALM	EMRHP-----	---NSVQEER	838
MMNOS	EEEQLLVVT	STFGNGDCPS	NGQTLKKSLS-	-----	-----	603
DNOS	KSFMKASSRQ	EFMKLPLQQV	KRIDRWDSL	GSTSDTFTEE	TFGPLSNVRF	813
BENOS	KSYK---IR-	-FNSVS-CSD	PLVSSWRRKR	KESNT---D	SAGALGTLRF	649
RNNOS	KSYK---VR-	-FNSVS-SYS	DSRKSSGDGP	DLRDNF---E	STGPLANVRF	879
MMNOS	--FM---LR-	-----	-----	ELNH-----	-----TFRY	615
-----FMN-----						
DNOS	AVFALGSSAY	PNFCAFQYV	DNILGELGGE	RLLRVAYGDE	MCGQEQSFRK	863
BENOS	CVFGLGSRAY	PHFCAFARAV	DTRLEELGGE	RLQLQGQDE	LCCQEEAFRG	699
RNNOS	SVFGLGSRAY	PHFCAFHAV	DTLLEELGGE	RILKMREGDE	LCCQEEAFRT	929
MMNOS	AVFGLGSSMY	PQFCFAHDI	DQKLSHLGAS	QLAPTGEDE	LSGQEDAFRS	665
DNOS	WAPEVFKLAC	ETFCLDPEES	--LSDASLAL	QNDSLTVNTV	RLVPSANKGS	911
BENOS	WAKAAFQASC	ETFCVGEEAK	--AAAQDIFS	PKRSWKRQRY	RLSAQAEGLO	747
RNNOS	WAKKVFKAAC	DVFCVGDVNV	IEXPNNLSIS	NDRSWKRNKF	RLTYVAEAPD	979
MMNOS	WAVQTFRAAC	ETFDVRSKHH	--IQIPKRFT	SNATWEPQQY	RLIQSPEPLD	713
DNOS	LDSSLSKYHN	KKVHCCKAKA	KPH-NLTRLS	EGAKTMMLE	ICAPGLEYP	960
BENOS	LLPGLIHVHR	RKMFOATVLS	VENLQSSKST	RATILVRLDT	AGQEGLQYQP	797
RNNOS	LTQGLSNVHK	KRVSAARLLS	RQNLQSPKFS	RSTIFVRLHT	NGNQELQYQP	1029
MMNOS	LNPALSSIHA	KNVFTMLRKS	QQNLQSEKSS	RTLLVQLTF	EGSRGPSYLP	763
-----FAD·PPI-----						
DNOS	GDKVGIFPAN	RTELVDGLLN	RLVGVDNPDE	VLQLQLLKEK	QTSNGIFKCV	1010
BENOS	GDHIGISAPN	RPGLVEALLS	RVEDPPPTE	SVAVEQL-EK	GSPGGPPPSW	846
RNNOS	GDHLGVFFGN	HEDLVNALIE	RLEDAPPANH	VVKVEMLEER	NTALGVISNW	1079
MMNOS	GEHLGIFPGN	QTALVQGILE	RVVDCPTPHQ	TVCLEVLDL	G-----SYW	807
DNOS	EPHDKIPPDT	LRNLLARFFD	LTPPSRQLL	TLLAGFCEDT	ADKERLELLV	1060
BENOS	VRDPRLPPCT	VRQALTFFLD	ITSPSPRLL	RLSTLAEPP	SEQELETLS	896
RNNOS	KDESRLPPCT	IFQAFKYLD	ITTPPTPLQL	QQFASLATNE	KEKQRLVLS	1129
MMNOS	VKDKRLPPCS	LSQALTYFLD	ITTPPTQLQL	HKLARFATDE	TDRQRLEAL	857
-----FAD·ISO-----						
DNOS	NDSSAYEDWR	HWRLPHLLDV	LEEFPSRPP	APLLLAQLTP	LQPRFYSISS	1110
BENOS	QDPRRYEWEK	LVRCPPTLLEV	LEQFPSVALP	APLLLTQLPL	LQPRYYSVSS	946
RNNOS	KGLQEYEEWK	WGKNPTMVEV	LEEFPSIQMP	ATLLLTQLSL	LQPRYYSISS	1179
MMNOS	Q-PSEYNDWK	FSMNPTFLEV	LEEFPSLHVP	AAFLLSQLPI	LKPRYYSISS	906

FIG. 16B

DNOS	SPRRVSDEIH	LTVAIVKYRC	EDGQGDERYG	VCSNYLSGLR	ADDELFMFVR	1160
BENOS	APNAHPGEVH	LTVAVLAYRT	QDGLGPLHYG	VCSTWLSQLK	TGDPVPCFIR	996
RNNOS	SPDMYPDEVH	LTVAIVSYHT	RDGEGPVHHG	VCSSWLNRIQ	ADDWVPCFVR	1229
MMNOS	SQDHTPSEVH	LTVAVVTYRT	RDGQGPLHHG	VCSTWIRNLK	PQDPVPCFVR	956
-----NADPH-Ribose-----						
DNOS	SALGFHLPSD	RSRPIILIGP	GTGIAPFRSF	WQEFQVLRDL	DPTAKLPKMW	1210
BENOS	GAPSFRLPPD	PYVPCILVGP	GTGIAPFRGF	WQE-RLHDIE	SKGLQPHPMT	1045
RNNOS	GAPSFHLPRN	PQVPCILVGP	GTGIAPFRSF	WQQ-RQFDIQ	HKGMNPCPMV	1278
MMNOS	SVSGFQLPED	PSQPCILIGP	GTGIAPFRSF	WQQ-RLHDSQ	HKGLKGG RMS	1005
DNOS	LFFGCRNRDV	D-LYAEKAE	LQKDQILDRV	FLALSREQAI	PKTYVQDLIE	1259
BENOS	LVFGCRCSQL	DHLYRDEVQD	AQERGVFGRV	LTAFSREPDS	PKTYVQDILR	1095
RNNOS	LVFGCRQSKI	DHIYREETLQ	AKNKGVFREL	YTAYSREPDR	PKKYVQDVLQ	1328
MMNOS	LVFGCRHPEE	DHLYQEEMQE	MVRKRVL FQV	HTGYSRLPGK	PKVYVQDILQ	1055
-----NADPH-Ade-----						
DNOS	QEF-DSLYQL	IVQERGHYV	CGDVTMAEHV	YQTIRKCIAG	KEQKSEAEVE	1308
BENOS	TELAAEVHRV	LCLERGHMFV	CGDVTMATS V	LQTVQRILAT	EGDMELDEAG	1145
RNNOS	EQLAESVYRA	LKEQG GHIYV	CGDVTMAADV	LKAIQRIMTQ	QGKLSEEDAG	1378
MMNOS	KQLANEVLSV	LHGEQGHLYI	CGDVRMARDV	ATTLK KLVAT	KLNLSEEQVE	1105
DNOS	TFLTLRDES	RYHEDIFGIT	LRTAEI----	--HTKSRATA	RIRMAS----	1348
BENOS	DVIGVLRDQQ	RYHEDIFGLT	LRTQEVTSRI	RTQSFSLQER	HLRGAVP WAF	1195
RNNOS	VFISRLRDDN	RYHEDIFGVT	LRTYEVTNRL	RSESI AFIEE	SKKDADE-VF	1427
MMNOS	DYFFQLKSQK	RYHEDIFGAV	F-SYGA----	-KKGSALEEP	--KAT-----	1142
DNOS	-----QP					1350
BENOS	DPPGPDTPGP					1205
RNNOS	-----SS					1429
MMNOS	-----RL					1144

FIG. 16C

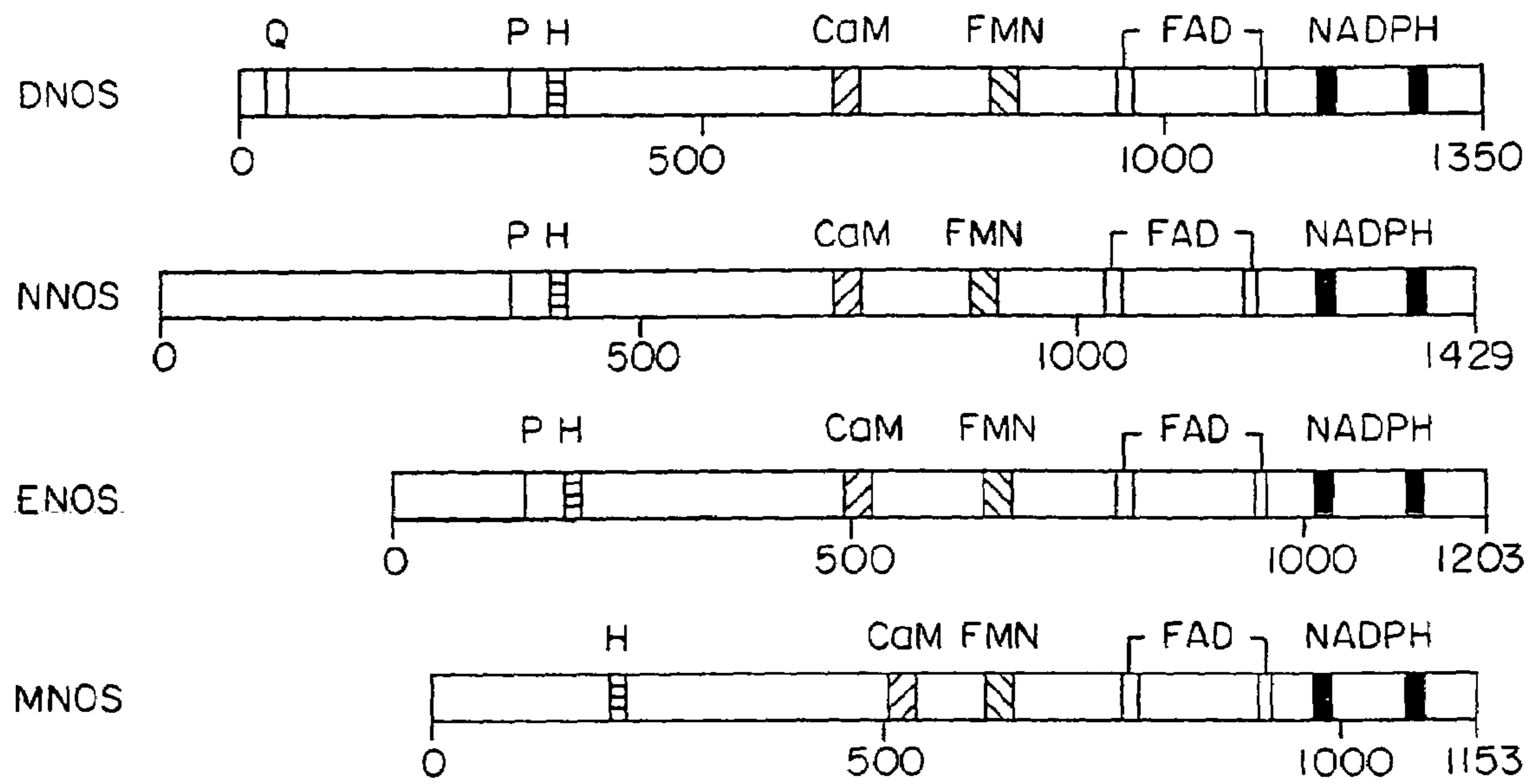
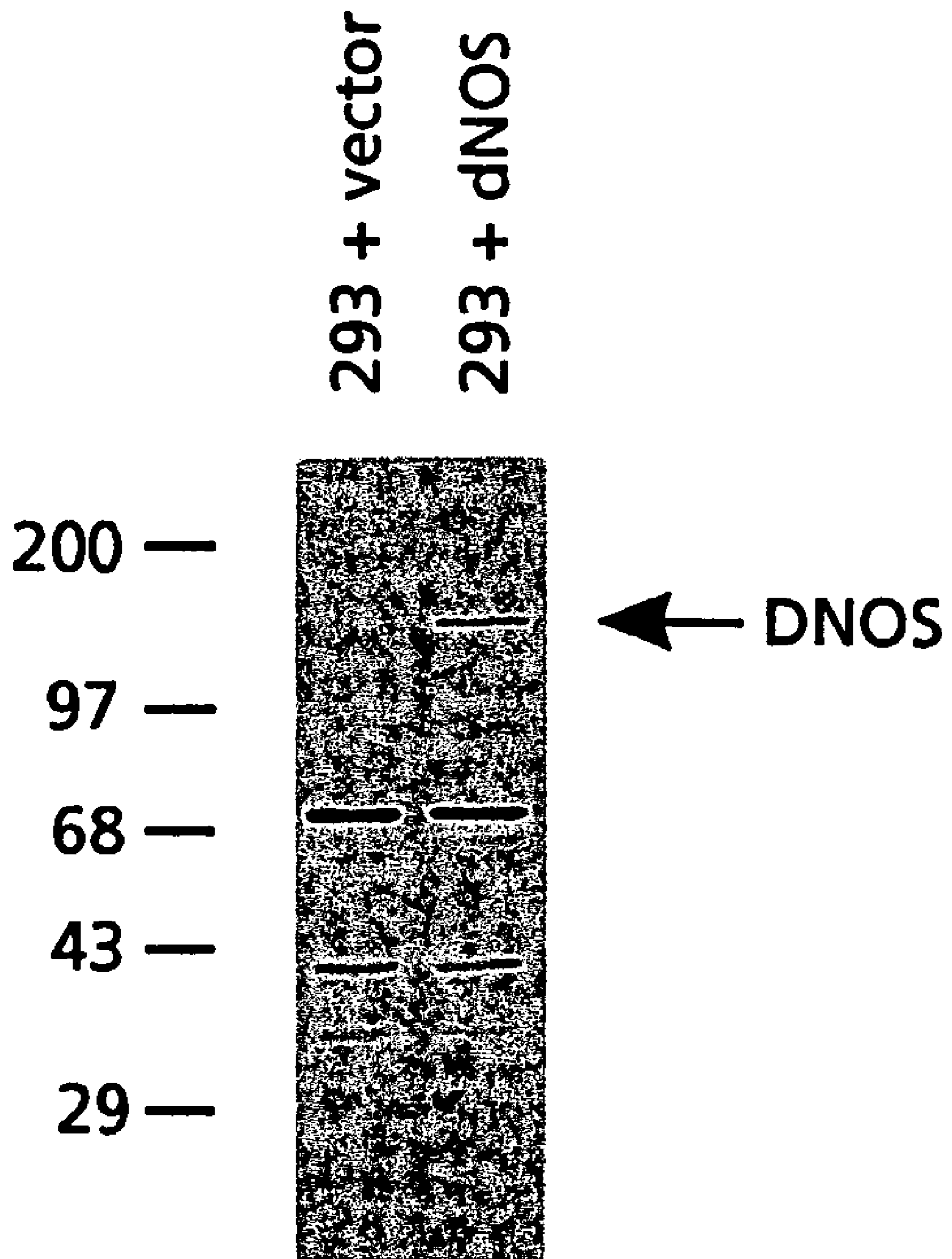


FIG. 16D



**FIG. 17A**

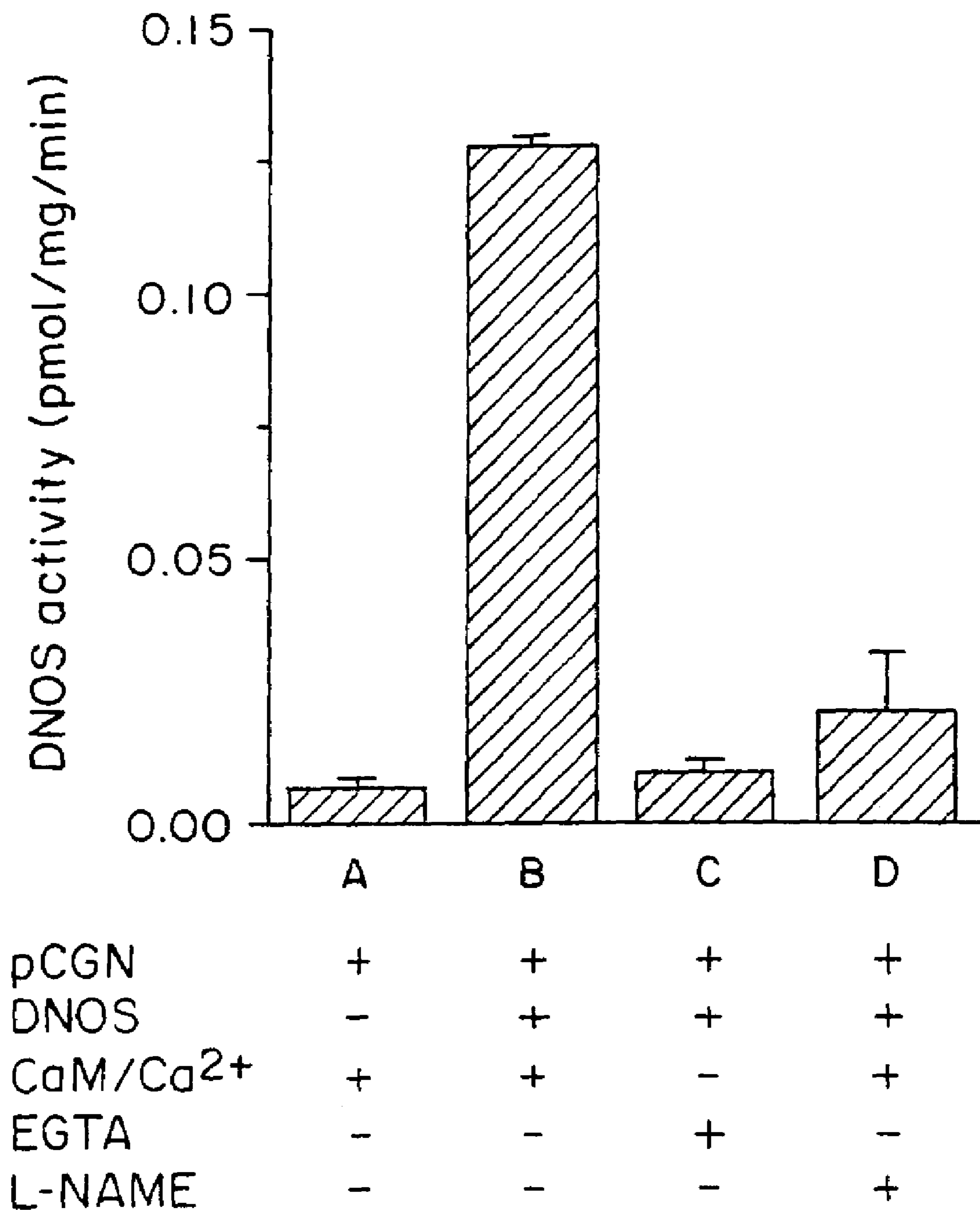


FIG. 17B

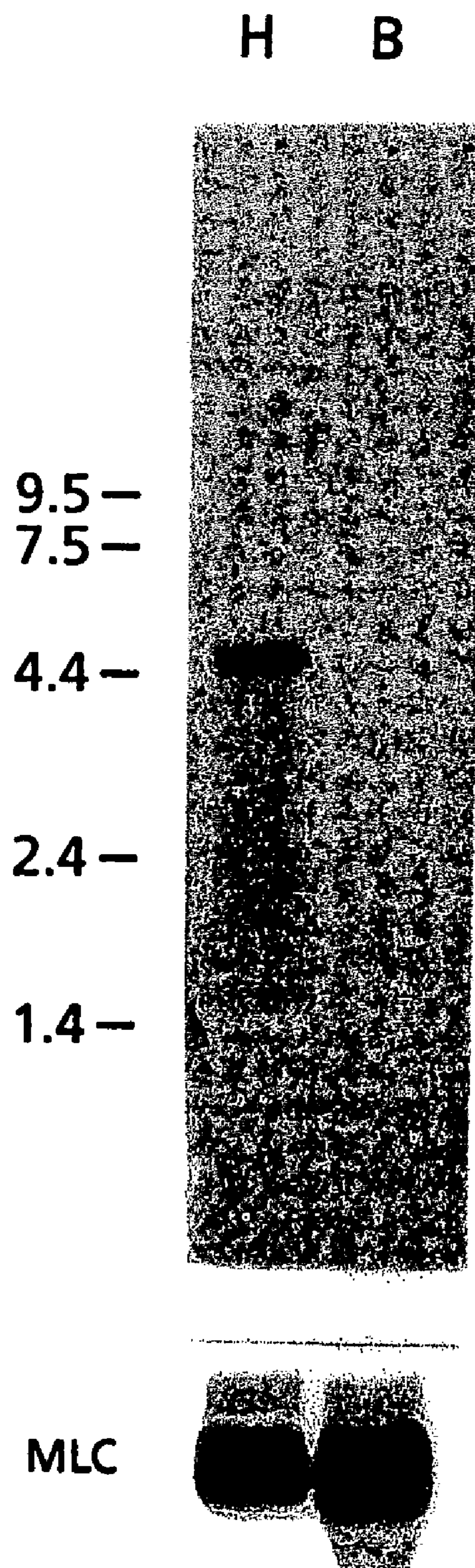
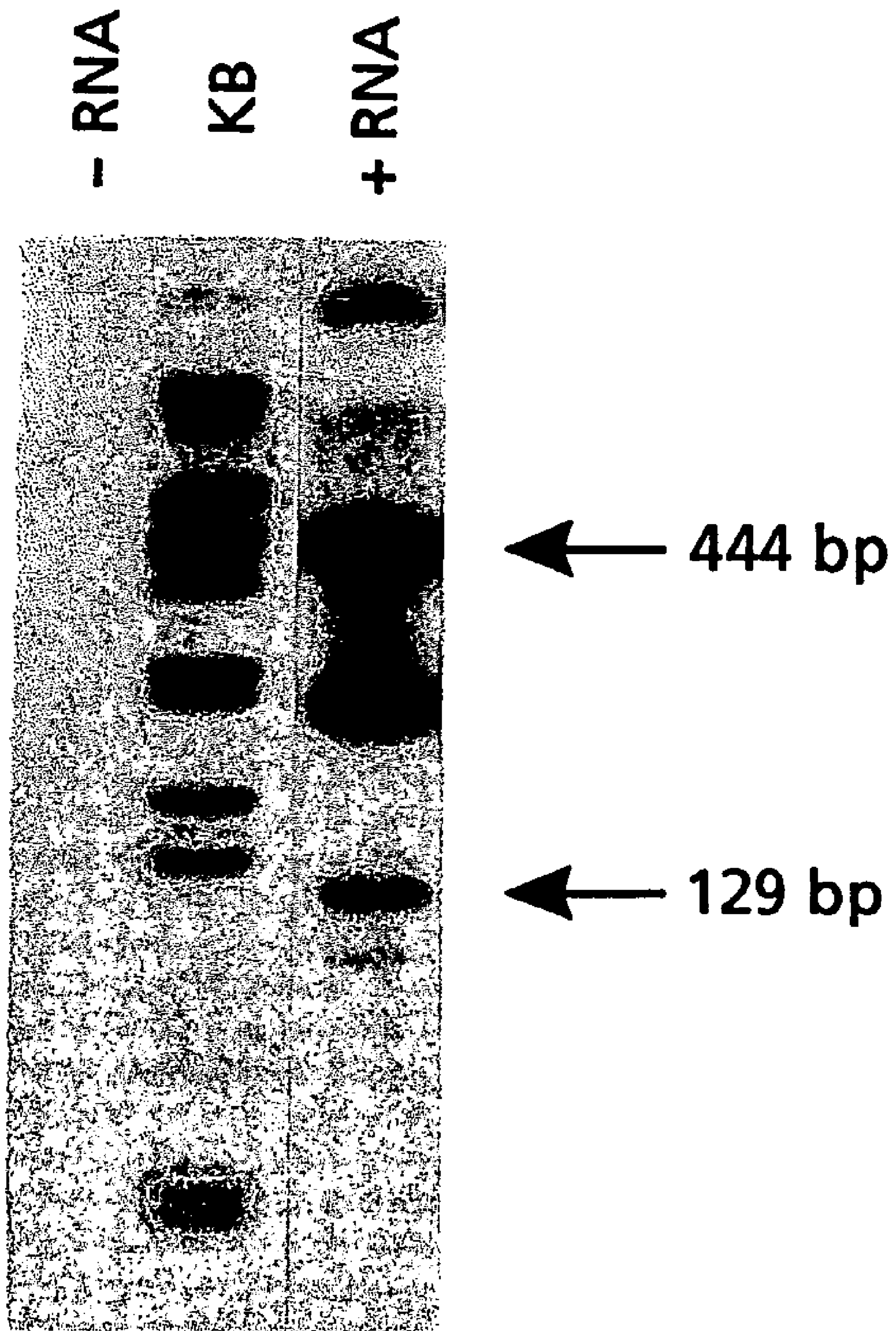


FIG. 18A



**FIG. 18B**

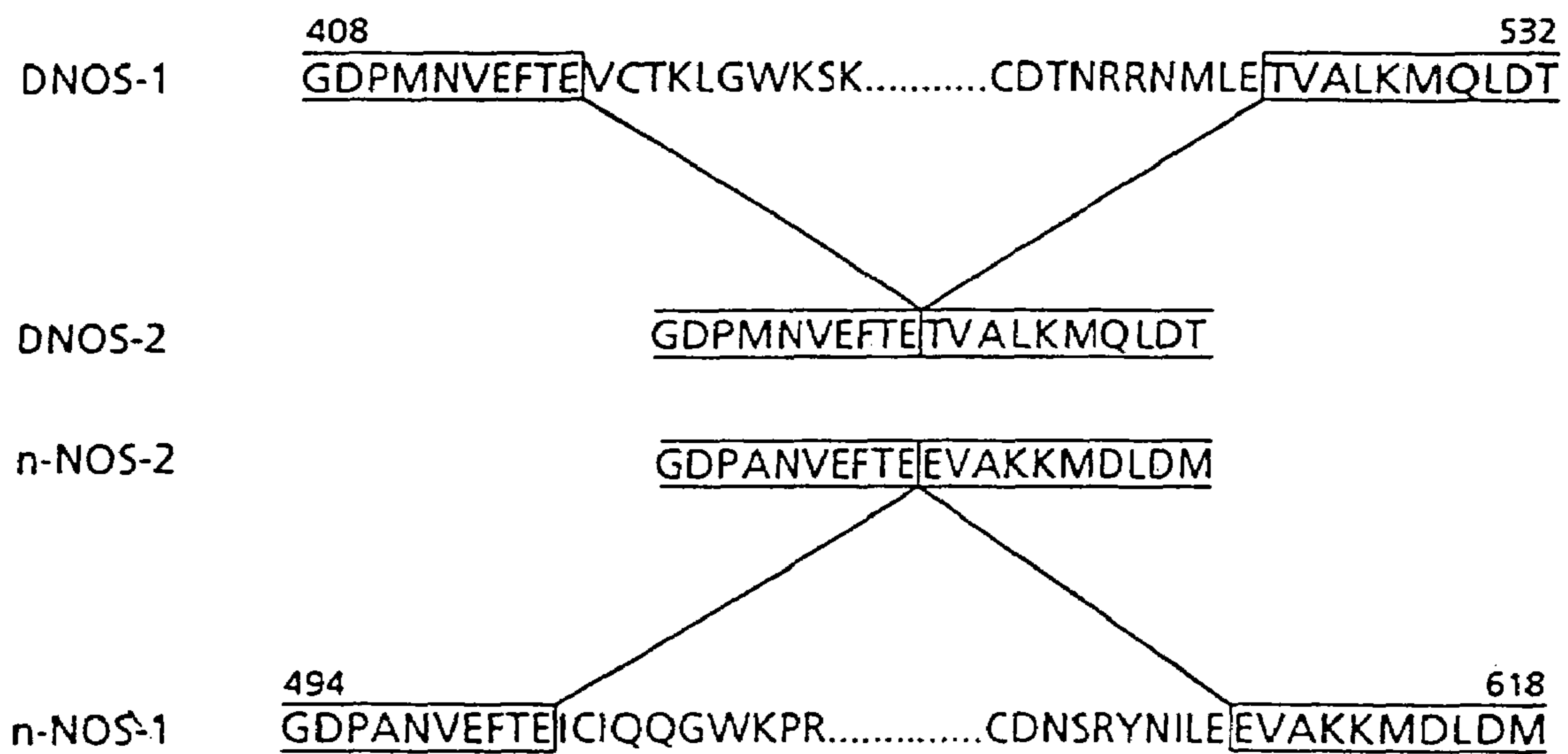


FIG. 18C



GAATTCGGTTTTTGGAAAAGTGAAGCAATTGAGTGC GGCCCGAAAAAGAGAGCCGCA  
GAAAGTTTGC GAACAGAATTTAATCAAAA ACTTGGAGGGTAAATTGTCCAAGTGGT  
TCACCTGTTGGCTGCATTTTAAATCAACGAGGCAAACAATCAGCGCAGAGGAGCTG  
CTCCACGTTCCCGGACAAGATGTCGCAGCATTTCACATCGATATTTGAGAACCTGC  
GATTCGTGACCATCAAACGTGCGACAAATGCGCAACAGCAACAGCAGCAGCAGCAG  
CAACAGCAACTTCAGCAGCAGCAGCAGCAGCTGCAGCAACAGAAGGCACAGACAC  
AGCAACAAAATAGCAGAAAAATCAAACTCAAGCAACGCCAACGTTGAATGGCAAT  
GGGCTCTTGAGCGGCAATCCAAATGGCGGAGGCGGTGACTCCTCGCCAGCCATGA  
AGTGGACCATCCGGGTGGAGCACAAGGAGCTCAAGCAGCAGGAGGCTTGCCATCTT  
TAAGTGGCACGCCATTGAGGCACCACAAGCGCGCCAGTATCTCCACAGCATCGCCTC  
CAATTCGCGAACGGCGTGGCACCACACCAGCATCGTGGTCGAACTGGATGGCAGT  
GGCAGCGGGAGTGGGAGTGGCGGTGGTGGCGTTGGCGTTGGTCAGGGTGC GGGTTG  
TCCTCCCTCGGGCAGCTGCACTGCGTCCGAAAAAGTTCCGCGGAACTATCGCCGTC  
GCCGAAAAACCAACAGCAGCCAGAAAGATGTCACAGGATTATCGGTCGCGTGCCG  
GCAGCTTTATGCACCTGGACGACGAGGGACGCAGTCTGCTGATGCGCAAGCCGATG  
AGACTGAAGAACATCGAGGGCAGGCCGGAGGTCTACGACACGCTGCACTGCAAGGG  
TCGCGAGATTTCTTCTGCTCGAAGGCCACCTGTACGAGCAGCATTATGAACATTGG  
CAATGCGGCGGTGGAGGCCAGGAAATCCGATCTGATCCTCGAACACGCCAAGGACT  
TCCTCGAGCAGTACTTTACATCGATAAAGCGTACATCATGTACCGCCACGAGACGC  
GATGGAAACAGGTGCGCCAGAGCATTGAGACCACTGGACACTATCAGCTAACCGAA  
ACGGAGCTAATTTATGGTGCCAAATTGGCCTGGCGCAATTCTTCACGTTGCATTGGC  
CGAATACAATGGTTCGAAGTTGCAGGTCTTTGACTGTCGTTATGTGACAACAACAAGT  
GGCATGTTTGAAGCCATTTGCAATCACATTAATATGCAACAAATAAGGGCAACCTG  
AGATCGGCCATCACGATATTTCCACAACGCACAGATGCCAAGCATGATTATCGCATT  
TGGAATAACCAATTAATATCTTATGCCGGCTACAAGCAGGCCGGATGGAAAAATCAT  
TGGCGATCCCATGAATGTGGAGTTTACAGAGGTCTGCACCAAGCTGGGCTGGAAGA  
GCAAGGGCAGCGAGTGGGACATACTGCCATTTGGTGGTCTCGGCCAATGGTCAAGAT  
CCGGACTACTTTGATTACCCGCCCGAATTGATACTGGAAGTTCCGCTGACCCATCCC  
AAATTCGAATGGTTCTCGGATCTGGGACTGCGATGGTACGCCCTGCCCGCCGTATCC  
AGTATGCTGTTGATGTGGGCGGCATTCAGTTTACGGCCACCACATTCAGTGGTTGG  
TACATGTGACAGAGATTGGCAGCCGGAATTTATGCGACACAAATCGCCGCAATAT  
GCTGGAGACGGTGGCGCTGAAGATGCAACTGGACACCCGTACGCCACATCCTTGT  
GGAAGGACAAGGCTGTGGTGGAGATGAACATTGCCGTGCTCCACTCCTACCAGAGT  
CGAACGTGACCATTGTGGATCACACACGGCCAGCGAGAGCTTTATGAAGCATTTTC  
GAGAACGAGTCCAAGCTCAGGAATGGGTGTCCCGCTGATTGGATTTGGATCGTGCC  
GCCGCTGTCGGGCTCCATAACGCCGGTATTCCATCAGGAGATGGCTCTGTACTACCT  
GAAGCCCTCGTTCGAGTACCAGGATCCCGCCTGGCGAACCCACGTGTGGAAAAAGG  
GGCGTGGCGAGAGCAAGGGCAAGAAGCCAAGACGTAAATTC AATTTTAAACAAATC  
GCTAGGGCTGTGAAATTTACATCGAACTATTTGGACGCGCCTTATCGAAACGCATA  
AAGGCAACAGTTCTATATGCCACCGAACTGGCAAATCGGAGCAGTATGCGAAGCA  
ACTTTGTGAACTCCTAGGGCACGCATTC AATGCACAGATATATTGCATGTCCGACTA  
CGATATATCCTCCATTGAGCACGAGGCATTGTTAATTGTTGTGGCCTCCACCTTTGGC  
AACGGTGATCCCCCGAAAACGGCGAGCTTTTCTCCAGGAATTGTATGCGATGCGT  
GTCCAGGAGTCTTCCGAGCATGGATTGCAGGACTCCAGCATTGGCTCGTCAAAGTCC  
TTCATGAAGGCCAGCTCGCGGCAGGAGTTCATGAAGCTGCCACTGCAACAGGTGAA  
GAGAATCGACCGATGGGACTCGCTGCGGGGCTCCACCTCGGACACCTTCACCGAGG  
AGACCTTTGGTCCCCTCTCCAATGTCCGGTTTGCCGTTTTTGCCTCGGCTCCTCGGC  
CTATCCAAATTTCTGCGCCTTCGGT CAGTATGTGGACAACATTCTGGGCGAGCTGGG  
CGGCGAACGCCTGCTGAGGGTGGCCTACGGCGACGAGATGTGCCGACAGGAGCAGT  
CGTTCCGGAAGTGGGCGCCCGAGGTATTCAAGTTGGCCTGCGAGACCTTCTGCCTGG  
ATCCAGAGGAGAGCCTTTCCGATGCCTCGCTAGCCCTGCAGAACGATTCGCTGACTG  
TGAATACGGTGC GCCTGGTGCCGTCGGCGAATAAGGGATCCCTGGACAGCAGTTTAT  
CCAAGTACCACAACAAGAAGGTGCACTGCTGCAAGGCCAAGGCCAAGCCCCACAAT  
TTGACCCGTTTGAGTGAGGGAGCCAAGACAACGATGCTGCTGGAGATCTGTGCACCT

FIG. 19A

GGCTTGGAGTACGAGCCGGGTGATCATGTGGGCATCTTTCCGGCGAATCGAACGGA  
ACTGGTTCGACGGACTGCTAAATCGACTGGTGGGTGTGGATAATCCCGACGAGGTGC  
TGCAGTTGCAATTGCTAAAGGAAAAGCAGACATCGAATGGTATATTCAAGTGCTGG  
GAGCCGCACGACAAAATACCGCCGGATACTCTAAGGAATCTACTGGCCCGATTCTTT  
GATCTGACCACTCCGCCATCGCGACAGCTACTCACCTGCTGGCTGGATTCTGTGAG  
GACACCGCGGACAAGGAGCGGCTGGAGTTGCTGGTCAACGATTTCGTCGGCCTACGA  
GGACTGGCGGCACTGGCGGCTGCCGCACCTGCTGGACGTCCTCGAGGAGTTCCTTC  
GTGCCGACCACCGGCTCCCCTTCTGCTTGCCCAACTAACGCCGCTGCAGCCTCGCTT  
CTATTCCATTTCTCGTCGCCGCGCCGCGTTAGTGACGAAATCCACCTGACGGTGGC  
CATCGTGAAGTACCGTTGTGAAGATGGTCAGGGTGACGAGCGGTACGGCGTGTGCT  
CTAACTATCTATCCGGCTTGCGGGCAGACGACGAGCTGTTTCATGTTTCGTGAGAAGCG  
CCTGGGCTTCCATTTGCCAGCGATCGGAGTCGTCCCATTATTCTGATTGGTCCTGG  
CACAGGAATAGCTCCATTTCCGCTCCTTTTGGCAGGAGTTCAGGTGCTACGCGACCT  
TGATCCCACGGCCAAATTGCCCAAGATGTGGCTCTTCTTTGGCTGCCGGAATCGGGA  
TGTGGACTTGTACGCCGAGGAGAAGGCAGAGCTACAGAAGGATCAAATCCTAGACC  
GAGTTTTTCTCGCTCTGTCCAGGGAGCAGGCCATTCCGAAGACATATGTGCAGGACC  
TGATTGAGCAGGAATTCGATTCGTTGTACCAGTTGATTGTCCAGGAGCGGGGCCACA  
TCTACGTCTGCGGCGATGTCACAATGGCCGAGCATGTGTACCAGACCATCAGGAAGT  
GCATTGCCGGCAAAGAGCAGAAAAGCGAGGCCGGAAGTTGAGACATTTTTTGCTAACA  
CTGCGGGACGAAAGTCGCTACCACGAGGACATCTTTGGCATCACGCTGCGAACGGC  
TGAGATACACACAAAGTCAAGGGCCACGGCCAGGATACGAATGGCCTCCCAGCCCT  
AAGGATAGATATTCGAAGTAATCAAAATAGGAGGGTGACATATCCAAATTCGAGAG  
GAATACCAAGCACTTGCTCTTTTTTTCTTCCATATTCAAATGCAATTAAATATTGTC  
GCTTTGTTTCATTACATATTCGTATGAATAACGTTTAAATAAATTACATTTTATTATTG  
ATTCTAATGTACAAATCAATTGTGAAATCAAAATCTAAATGTTAAATATATTTCAA  
ATAAACGAATCGAAAAGGAATTC

FIG. 19B

## METHOD FOR ASSESSING THE EFFECT OF A DRUG ON LONG TERM MEMORY FORMATION

### RELATED APPLICATIONS

This application is a Continuation of U.S. application Ser. No. 09/419,371 filed Oct. 14, 1999, now U.S. Pat. No. 6,890,516 which is a Divisional of U.S. application Ser. No. 08/809,917 filed Jul. 7, 1997, now U.S. Pat. No. 6,689,557, which is the U.S. National Phase of International Application No. PCT/US95/13198, filed Oct. 6, 1995, which is a Continuation-in-Part of U.S. application Ser. No. 08/361,063, filed Dec. 21, 1994, now U.S. Pat. No. 6,051,559, which is a Continuation-In-Part of U.S. application Ser. No. 08/319,866, filed Oct. 7, 1994, now U.S. Pat. No. 5,929,223. The entire teachings of the above applications are incorporated herein by reference.

This application contains an electronically submitted substitute sequence listing text file which has been filed through the EFS-Web. The materials submitted in the text file and substitute paper copy of the substitute sequence listing are incorporated herein by reference in their entirety. The text file contains a single file named "17VV7191.txt" (70KB, created on June 17, 2009). The text file was created on Jun. 17, 2009.

### GOVERNMENT SUPPORT

This invention was made with Government support under Grant No. 1R01 HD32245-01 awarded by the National Institutes of Health. The United States government has certain rights in this invention.

### BACKGROUND OF THE INVENTION

Activation of the cyclic 3',5'-adenosine monophosphate (cAMP) signal transduction pathway can have long-lasting global consequences through its influence on the expression of specific genes. This is true for simple organisms as well as mammals, where many of the known cAMP-responsive genes can have important neural and endocrine roles. Additional information regarding activation of this pathway would be useful, particularly as this activation pertains to the ability of animals to remember activities or events.

### SUMMARY OF THE INVENTION

The present invention is based on Applicants' discovery of the dCREB1 and dCREB2 genes. The present invention is further based on Applicants' discovery that the *Drosophila* CREB2 gene codes for proteins of opposite functions. One isoform (e.g., dCREB2-a) encodes a cyclic 3',5'-adenosine monophosphate (cAMP)-responsive transcriptional activator. Another isoform (e.g., dCREB2-b) codes for an antagonist which blocks the activity of the activator.

When the blocking form is placed under the control of the heat-shock promoter, and transgenic flies are made, a brief shift in temperature induces the synthesis of the blocker in the transgenic fly. This induction of the blocker (also referred to herein as the repressor) specifically disrupts long-term, protein synthesis dependent memory of an odor-avoidance behavioral paradigm.

As a result of Applicants' discovery, a method is herein provided to regulate long term memory in an animal. The method of regulating long term memory described herein comprises inducing expression of a dCREB2 gene or a fragment thereof in the animal.

The dCREB2 gene encodes several isoforms. Examples of an isoform encoded by the dCREB2 gene are dCREB2-a, dCREB2-b, dCREB2-c, dCREB2-d, dCREB2-q, dCREB2-r and dCREB2-s.

5 The isoforms encoded by the dCREB2 gene include cAMP-responsive activator isoforms and antagonistic blocker (or repressor) isoforms of the activator isoforms. Cyclic AMP responsive activator isoforms can function as a cAMP-responsive activator of transcription. Antagonistic  
10 repressors can act as a blocker of activators. An example of a cAMP-responsive activator isoform is dCREB2-a. An example of an antagonistic repressor (or blocker) isoform is dCREB2-b. The terms blocker and repressor are used interchangeably herein.

15 In one embodiment of the invention, the dCREB-2 gene encodes a cAMP-responsive activator isoform and inducing said gene results in the potentiation of long term memory.

Alternatively, inducing the dCREB2 gene encoding a cAMP-responsive activator isoform activates the production  
20 of a protein which is necessary for the formation of long term memory.

In another embodiment of the invention, the dCREB2 gene encodes a repressor isoform and inducing said gene results in the blocking of long term memory.

25 A further embodiment of the invention relates to a method of regulating long term memory in an animal comprising inducing repressor and activator isoforms of dCREB2 wherein long term memory is potentiated in the animal when the net amount of functional activator ( $\Delta C$ ) is greater than  
30 zero.

The invention also relates to a method of identifying a substance capable of affecting long term memory in an animal comprising the determination that said substance alters the induction or activity of repressor and activator isoforms of  
35 dCREB2 from normal in the animal.

As referred to herein, an activator isoform includes dCREB2-a and functional fragments thereof and a repressor isoform includes dCREB2-b and functional fragments thereof.

40 Other embodiments of the invention relate to a method of enhancing long term memory formation in an animal comprising increasing the level of activator homodimer from normal, decreasing the level of activator-repressor heterodimer from normal, or decreasing the level of repressor homodimer  
45 from normal in the animal.

Still another embodiment of the invention relates to a method of identifying a substance capable of affecting long term memory in an animal comprising the determination that said substance alters activator homodimer, activator-repressor heterodimer and/or repressor homodimer formation from  
50 normal in the animal.

As referred to herein, an activator homodimer includes the dCREB2a homodimer, an activator-repressor heterodimer includes the dCREB2a-dCREB2b heterodimer, and a repressor  
55 homodimer includes the dCREB2b homodimer.

A further embodiment of the invention relates to isolated DNA encoding a cAMP responsive transcriptional activator. Such a cAMP responsive transcriptional activator can be encoded by a *Drosophila* dCREB2 gene or by homologues or  
60 functional fragments thereof. For example, a cAMP responsive transcriptional activator can be encoded by the dCREB2 gene which codes for dCREB2-a or by a gene encoded by the sequences presented herein.

Still another embodiment of the invention relates to isolated DNA encoding an antagonist of cAMP-inducible transcription. Such an antagonist of cAMP-inducible transcription can be encoded by a *Drosophila* dCREB2 gene or by

homologues or functional fragments thereof. For example, an antagonist of cAMP-inducible transcription can be encoded by the dCREB2 gene which codes for dCREB2-b.

Another embodiment of the invention relates to isolated DNA (SEQ ID NO.: 25) which encodes a *Drosophila* dCREB2 gene or functional fragments thereof.

A further embodiment of the invention relates to isolated DNA encoding an enhancer-specific activator. Such an enhancer-specific activator can be encoded by a *Drosophila* dCREB1 gene or by homologues or functional fragments thereof.

Another embodiment of the invention relates to isolated DNA encoding a nitric oxide synthase of *Drosophila* (DNOS). Such DNA can encode a DNOS of neuronal locus. The DNOS encoded can contain, for example, putative heme, calmodulin, flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD) and nicotinamide adenine dinucleotide phosphate, in its reduced form, (NADPH) binding site domains.

A further embodiment of the invention relates to a method for assessing the effect of a drug on long term memory formation comprising administering the drug to *Drosophila*, subjecting the *Drosophila* to classical conditioning to at least one odorant and electrical shock, and assessing the performance index of the classical conditioning, wherein the effect of the drug occurs when it alters the performance index from normal. The drug can affect long term memory formation by, for example, altering the induction or activity of repressor and activator isoforms of dCREB2.

A still further embodiment of the invention relates to the assessment that an animal will have an enhanced or, alternatively, a diminished capability of possessing long term memory. This assessment can be performed by determining the amount of cAMP-responsive activator isoforms, cAMP-responsive repressor or blocker isoforms, or dimers of these isoforms that are present in the animal, where these isoforms are encoded by the CREB2 or a homologous gene. Enhanced capability of possessing long term memory will be more likely as the amount of activator exceeds the amount of repressor, i.e. in direct proportion to the size of the net amount of functional activator ( $\Delta C$ ) when this quantity is greater than zero. Conversely, diminished capability of processing long term memory will be more likely as the amount of repressor exceeds the amount of activator, i.e. in direct proportion to the size of the net amount of functional activator ( $\Delta C$ ) when this quantity is less than zero.

Another embodiment of the invention relates to a screening assay of pharmaceutical agents as enhancers of long term memory or as obstructors of long term memory in animals. The screening assay is performed by determining the change in the amount of cAMP-responsive activator isoforms, cAMP-responsive repressor or blocker isoforms, or dimers of these isoforms that is present in an animal or, more preferably, in a cell culture system or in *Drosophila* when the pharmaceutical agent is present, in comparison to when the pharmaceutical agent is not present, where these isoforms are encoded by the CREB2 or a homologous gene. Enhancers of long term memory cause a net increase in the amount of activator isoforms relative to the amount of repressor isoforms, i.e. an increase in the net amount of functional activator ( $\Delta C$ ). Obstructors of long term memory cause a net decrease in the amount of activator isoforms relative to the amount of repressor isoforms, i.e. a decrease in the net amount of functional activator ( $\Delta C$ ). The pharmaceutical agent can cause these changes by acting, for example, to alter the expression (transcription or translation) of the respective activator and/or repressor isoforms from the CREB2 or a

homologous gene, to alter the formation of activator homodimers, activator-repressor heterodimers and/or repressor homodimers from the expressed isoforms, or to alter the interaction of one or more of these isoform or dimer types at their molecular targets. The long term memory activator isoform/repressor isoform system herein disclosed provides a unique platform for conducting such screening assays.

A further embodiment of the invention relates to an assay of pharmaceutical agents for their property as facilitators or hinderers of long term memory in animals. The assay is performed by administering the pharmaceutical agent to *Drosophila* prior to subjecting the *Drosophila* to a Pavlovian olfactory learning regimen. This regimen assesses the long term memory capabilities of the *Drosophila* by subjecting the flies to a massed and/or a spaced training schedule. Transgenic lines of these flies containing altered dCREB2 genes can be used to further elucidate the long term memory facilitation or hindering property of the pharmaceutical agent. The assay provides data regarding the acquisition of long term memory by the *Drosophila* after exposure to the pharmaceutical agent. These data are compared to long term memory acquisition data from *Drosophila* that have not been exposed to the pharmaceutical agent. If the exposed flies display faster or better retained long term memory acquisition than the unexposed flies, the pharmaceutical agent can be considered to be a facilitator of long term memory. Conversely, if the exposed flies display slower or less retained long term memory acquisition than the unexposed flies, the pharmaceutical agent can be considered to be a hinderer of long term memory. Since the genetic locus for this long term memory assay in *Drosophila* resides in the dCREB2 gene, the results from this assay can be directly applied to other animals that have homologous genetic loci (CREB2 or CREM genes).

#### BRIEF DESCRIPTION OF THE DRAWING

FIG. 1A depicts the DNA sequence (SEQ ID NO.: 1) and predicted amino acid sequence (SEQ ID NO.: 2) of the dCREB2-a coding region. The basic region and leucine zipper domains are indicated by solid and broken bold underlining, respectively; positively-charged residues in the basic region are circled; periodic leucines in the zipper motif are boxed; glutamines in the activation domain are underlined; the short amino acid motif with target sites for kinases, starting at residue 227, is indicated by a bold outline; and sequences specified by alternatively-spliced exons 2, 4 and 6 are shaded.

FIG. 1B depicts the amino acid sequences of the bZIP domains of dCREB2 (SEQ ID NO.: 3), mammalian CREB (SEQ ID NO.: 4), CREM (SEQ ID NO.: 5) and ATF-1 (SEQ ID NO.: 6). Differences between dCREB2 and CREB are boxed.

FIG. 2 is a schematic diagram of dCREB2 isoforms with the exon boundaries defined with respect to dCREB2-a. Diagram is not drawn to scale.

FIG. 3 is a bar graph representation of results showing pKA-responsive transcriptional activation by dCREB2-a.

FIG. 4 is a bar graph representation of results showing the transcriptional effect of dCREB2-b and a mutant variant on pKA-responsive activation by dCREB2-a.

FIG. 5 depicts the DNA sequence (SEQ ID NO.: 7) and predicted amino acid sequence (SEQ ID NO.: 8) of the dCREB1 coding region. The basic region and leucine zipper domains are indicated by solid and broken bold underlining, respectively; positively-charged residues in the basic region are circled; periodic leucines of the zipper motif are boxed; and in the acid-rich region of the activation domain, nega-

## 5

tively-charged amino acids are underlined and proline residues are indicated by diamonds.

FIG. 6 is a bar graph representation of results showing transcriptional activation of a CRE reporter gene by dCREB1 in *Drosophila* Schneider L2 cell culture.

FIG. 7A is a photomicrograph of a Northern blot depicting the effect of heat shock induction on dCREB2-b expression: wt=wildtype flies; CREB=17-2 transgenic flies; lanes 1-2: no heat shock; lanes 2-3: immediately after heat shock; lanes 5-6: three hours after heat shock.

FIG. 7B is a photograph of a Western blot depicting the effect of heat shock induction on dCREB2-b protein production: wt=wildtype flies; CREB=17-2 transgenic flies; lanes 1-2: no heat shock; lanes 2-3: immediately after heat shock; lanes 5-6: one hour after heat shock; lanes 7-8: three hours after heat shock; lanes 9-10: 9 hours after heat shock; lanes 11-12: 24 hours after heat shock.

FIG. 7C is a photograph of a Western blot depicting the effect of heat shock induction on dCREB2 and dCREB2-mLZ (a mutated dCREB2-b) protein production: wt=17-2 transgenic flies (expressing wildtype blocker, dCREB2-b); m=A2-2 transgenic flies (expressing mutant blocker, dCREB2-mLZ); lanes 1-2: no heat shock; lanes 3-4: immediately after heat shock; lanes 5-6: three hours after heat shock; lanes 7-8: six hours after heat shock.

FIG. 8 is a bar graph representation of results showing the effect of cycloheximide (CXM) feeding, before or after spaced or massed training, on one-day memory retention: stripped bars=+CXM; hatched bars=-CXM.

FIG. 9A is a bar graph representation of results showing the effect of heat shock induction on one-day memory retention in wildtype (Can-S) flies and hs-dCREB2-b transgenic (17-2) flies given spaced or massed training: hatched bars=wildtype (Can-S) flies; stripped bars: hs-dCREB2-b transgenic (17-2) flies; hs=heat shock.

FIG. 9B is a bar graph representation of results showing the effect of heat shock induction on one-day memory retention in wildtype (Can-S) flies or hs-dCREB2-b transgenic (M11-1) flies given spaced or massed training: hatched bars=wildtype (Can-S) flies; stripped bars: hs-dCREB2-b transgenic (M11-1) flies; hs=heat shock.

FIG. 9C is a bar graph representation of results showing the effect of heat shock induction on learning in wildtype (Can-S) flies and hs-dCREB2-b transgenic (17-2) flies given spaced or massed training: hatched bars=wildtype (Can-S) flies; stripped bars: hs-dCREB2-b transgenic (17-2) flies; hs=heat shock.

FIG. 10 is a bar graph representation of results showing the effect of heat shock induction on one-day memory retention in wildtype [w(isoCJ1)] flies, hs-dCREB2-b transgenic (17-2) flies, and mutant hs-dCREB2-mLZ transgenic (A2-2) flies given spaced training: hatched bars=wildtype [w(isoCJ1)] flies; stripped bars=hs-dCREB2-b transgenic (17-2) flies; white bars=mutant hs-dCREB2-mLZ transgenic (A2-2) flies; hs=heat shock.

FIG. 11 is a bar graph representation of results showing the effect of heat shock induction on seven-day memory retention (long term memory) in wildtype (Can-S) flies and hs-dCREB2-b transgenic (17-2) flies given spaced training: hatched bars=wildtype (Can-S) flies; stripped bars=hs-dCREB2-b transgenic (17-2) flies; hs=heat shock.

FIG. 12 is a bar graph representation of results showing the effect of heat shock induction on one-day memory retention in hs-dCREB2-b transgenic (17-2) flies, radish mutant flies, and radish hs-dCREB2-b double mutant (rsh; 17-2) flies given spaced training: hs=heat shock; hatched bars=-hs; stripped bars=+hs.

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FIG. 13A is a graphic representation of results showing the effect of repeated training sessions on seven-day memory retention (long term memory) in wildtype (Can-S) flies with long term memory as a function of the number of training sessions indicated by open circles and a negative accelerating exponential Gompertz (growth) function fit to the individual performance indexes (PIs) using a nonlinear iterative least squares method indicated by the solid line.

FIG. 13B is a graphic representation of results showing the effect of the rest interval between each training session on seven-day memory retention (long term memory) in wildtype (Can-S) flies with long term memory as a function of the rest interval indicated by open circles and a negative accelerating exponential Gompertz (growth) function fit to the individual performance indexes (PIs) using a nonlinear iterative least squares method indicated by the solid line.

FIG. 14 depicts a conceptual model of a molecular switch for the formation of long term memory based on differential regulation of CREB isoforms with opposing functions with  $\Delta C$  indicating the net effect of CREB activators.

FIG. 15A is a bar graph representation of results showing the effect of 48 massed training sessions (48x massed) or 10 spaced training sessions with a 15-minute rest interval (10x spaced) on seven-day memory retention in wildtype (Can-S) flies.

FIG. 15B is a bar graph representation of results showing the effect of one (1x), two (2x) or ten (10x) massed training sessions, three hours after heat-shock induction of the transgene (induced) or in the absence of heat-shock (uninduced), on seven-day memory retention in wildtype (Can-S) flies, hsp-dCREB2-a transgenic (C28) flies, and hsp-dCREB2-a transgenic (C30) flies: black bars=wildtype (Can-S) flies; stripped bars=hsp-dCREB2-a transgenic (C28) flies; and white bars=hsp-dCREB2-a transgenic (C30) flies.

FIG. 15C is a bar graph representation of results showing responses three hours after heat shock in wildtype (Can-S) flies and hsp-dCREB2-a transgenic (C28) flies to odors, either octanol (OCT) or methylcyclohexanol (MCH), or to shock (60 V DC): black bars=wildtype (Can-S) flies; stripped bars=hsp-dCREB2-a transgenic (C28) flies.

FIG. 16A-16C depict the deduced amino acid sequences of DNOS and mammalian NOSs with amino acid numbering starting at the first methionine in each open reading frame (ORF), putative binding domains for cofactors (overlined) demarcated as in previously published reports on mammalian NOSs, and amino acids which have been proposed as contacts with FAD and NADPH based on crystal structure of the ferredoxin NADP reductase (Karplus, P.A., *Science*, 251: 60-66 (1991)) conserved at equivalent positions (bullet points): DNOS, *Drosophila* NOS (SEQ ID NO. 9); RNNOS, rat neuronal NOS (SEQ ID NO. 11); BENOS, bovine endothelial NOS (SEQ ID NO. 10); MMNOS, mouse macrophage NOS (SEQ ID NO. 12). Sequence alignment and secondary structure predictions were performed by Genework 2.3 (IntellicGenetics).

FIG. 16D is a schematic diagram of the domain structure of *Drosophila* and mammalian NOS proteins with the proposed cofactor-binding sites for heme (H), calmodulin (CaM), flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), nicotinamide adenine dinucleotide phosphate (NADPH) and the glutamine-rich domain (Q) in DNOS shown.

FIG. 17A is a photograph of a Western blot showing DNOS expression in 293 human embryonic kidney cells.

FIG. 17B is a bar graph representation of results showing DNOS enzyme activity measured in 293 human embryonic kidney cell extracts by conversion of  $^3\text{H-L-arginine}$  to  $^3\text{H-L-}$

citrulline: in the presence of exogenous  $\text{Ca}^{2+}$  or calmodulin (group B); in the presence of 1 mM EGTA without exogenous  $\text{Ca}^{2+}$  or calmodulin (group C); in the presence of 100 mM L-NAME with exogenous  $\text{Ca}^{2+}$  or calmodulin (group D).

FIG. 18A is a photomicrograph of a Northern blot showing a 5.0 kb dNOS transcript present in *Drosophila* heads: H=head; B=body.

FIG. 18B is a photograph of an agarose gel stained with ethidium bromide showing the expression by the dNOS gene of two alternatively spliced mRNA species with the arrows indicating the positions of the DNA fragments of the expected sizes: the 444 bp long-form fragment and the 129 bp short-form fragment. The other bands present in the lane are artifacts from heteroduplexes that failed to denature. KB=size markers.

FIG. 18C depicts the alignment of the deduced amino acid sequence of two protein isoforms of DNOS and mouse neuronal NOS: top part shows the relation between two conceptual *Drosophila* NOS proteins, DNOS-1 (amino acid residues 408-427 and 513-532 of SEQ ID NO.: 9) and DNOS-2 (SEQ ID NO.: 14), corresponding to the longer and shorter RT-PCR products, respectively; the bottom part shows the relationship between the relevant regions of two protein isoforms of the mouse neuronal NOS, n-NOS-1 (amino acid residues 494-513 and 599-618; SEQ ID NO.: 13 and SEQ ID NO.: 15, respectively) and n-NOS-2 (SEQ ID NO.: 16); and the numbers indicate the positions of the amino acid residues relative to the first methionine in the respective OFRs.

FIG. 19A-19B depicts the nucleotide sequence (SEQ ID NO.: 25) of a dNOS cDNA encoding the DNOS protein. The open reading frame of 4050 bp starts at nucleotide 189 and ends at nucleotide 4248.

#### DETAILED DESCRIPTION OF THE INVENTION

Applicants have cloned and characterized two genes, designated dCREB2 and dCREB1, isolated through a DNA-binding expression screen of a *Drosophila* head cDNA library in which a probe containing three cAMP-responsive element (CRE) sites was used.

The dCREB2 gene codes for the first known cAMP-dependent protein kinase (PKA) responsive CREB/ATF transcriptional activator in *Drosophila*. A protein data base search showed mammalian CREB, CREM and ATF-1 gene products as homologous to dCREB2. For these reasons, dCREB2 is considered to be a member, not only of the CREB/ATF family, but of the specific cAMP-responsive CREB/CREM/ATF-1 subfamily. It is reasonable to expect that dCREB2 is involved in *Drosophila* processes which are analogous to those which are thought to depend on cAMP-responsive transcriptional activation in other animal systems.

Applicants have shown that the dCREB2 transcript undergoes alternative splicing. Splice products of dCREB2 were found to fall into two broad categories: one class of transcripts (dCREB2-a, -b, -c, -d) which employs alternative splicing of exons 2, 4 and 6 to produce isoforms whose protein products all have the bZIP domains attached to different versions of the activation domain and a second class of transcripts (dCREB2-q, -r, -s) which have splice sites which result in in-frame stop codons at various positions upstream of the bZIP domain. These all predict truncated activation domains without dimerization or DNA binary activity.

dCREB2-a, -b, -c and -d are splice forms that predict variants of the activation domain attached to a common basic region-leucine zipper. These alternative splice forms result in seemingly minor changes in the size and spacing of parts of the activation domain. Nevertheless, alternative splicing of

the activation domain has profound effects on the functional properties of dCREB2 products. Isoform dCREB2-a produces a PKA-responsive transcriptional activator in cell culture, whereas dCREB2-b, lacking exons 2 and 6, produces a specific antagonist. This dCREB2 splicing pattern (and its functional consequences) is virtually identical to that seen in the CREM gene. Similarly located, alternatively-spliced exons in the CREM gene determine whether a particular isoform is an activator or an antagonist (deGroot, R. P. and P. Sassone-Corsi, *Mol. Endocrinol.*, 7: 145-153 (1993); Foulkes, N. S. et al., *Nature*, 355: 80-84 (1992)).

The ability of the phosphorylation domain (KID domain) to activate in trans other constitutive transcription factors which are bound nearby could potentially transform a CREM antagonist (which contains the KID domain but is lacking an exon needed for activation) into a cAMP-responsive activator. Since the modular organization of these molecules has been conserved, dCREB2-d could have this property.

In contrast to the dCREB2 splicing variants that encode isoforms with a basic region-leucine zipper domain, the dCREB2-q, -r and -s splice forms incorporate in-frame stop codons whose predicted protein products are truncated before the bZIP region. Isoforms of this type have been identified among the products of the CREB gene (deGroot, R. P. and P. Sassone-Corsi, *Mol. Endocrinol.*, 7: 145-153 (1993); Ruppert, S. et al., *EMBO J*, 11: 1503-1512 (1992)) but not the CREM gene. The function of these truncated CREB molecules is not known, but at least one such CREB mRNA is cyclically regulated in rat spermatogenesis (Waeber, G. et al., *Mol. Endocrinol.*, 5: 1418-1430 (1991)).

So far, dCREB2 is the only cAMP-responsive CREB transcription factor isolated from *Drosophila*. Other *Drosophila* CREB molecules, BBF-2/dCREB-A (Abel, T. et al., *Genes Dev.*, 6: 466-488 (1992); Smolik, S. M. et al., *Mol. Cell Biol.*, 12: 4123-4131 (1992)), dCREB-B (Usui, T. et al., *DNA and Cell Biology*, 12(7): 589-595 (1993)) and dCREB1, have less homology to mammalian CREB and CREM. It may be that dCREB2 subsumes functions of both the CREB and CREM genes in *Drosophila*. The mammalian CREB and CREM genes are remarkably similar to one another in several respects. It has been suggested that CREB and CREM are the product of a gene duplication event (Liu, F. et al., *J. Biol. Chem.*, 268: 6714-6720 (1993); Riabowol, K. T. et al., *Cold Spring Harbor Symp. Quant. Biol.*, 1: 85-90 (1988)). dCREB2 has a striking degree of amino acid sequence similarity to the CREB and CREM genes in the bZIP domain. Moreover, comparison of alternative splicing patterns among CREB, CREM and dCREB2 indicates that dCREB2 generates mRNA splicing isoforms similar to exclusive products of both CREB and CREM. Taken together, the sequence information and the splicing organization suggest that dCREB2 is an ancestor of both the mammalian CREB and CREM genes.

As discussed further herein, one phenomenon in which dCREB2 might act with enduring consequences is in long-term memory. This possibility is a particularly tempting one because recent work in *Aplysia* indicates that a CREB factor is likely to function in long-term facilitation by inducing an "immediate early" gene (Alberini, C. M. et al., *Cell*, 76: 1099-1114 (1994); Dash, P. K., *Nature*, 345: 718-721 (1990)). Recent experiments with a conditionally-expressed dCREB2-b transgene indicate that it has specific effects on long-term memory in *Drosophila*.

The product of the second gene described herein, dCREB1, also appears to be a member of the CREB/ATF family. Gel-retardation assays indicate that it binds specifically to CREs. It has a basic region and an adjacent leucine zipper at its carboxyl end, but this domain shows limited amino acid

sequence similarity to other CREB/ATF genes. The presumed transcriptional activation domain of dCREB1 is of the acid-rich variety. Furthermore, it has no consensus phosphorylation site for PKA. dCREB1 can mediate transcriptional activation from CRE-containing reporters in the *Drosophila* L2 cell line, but this activation is not dependent on PKA.

A recurrent finding from work on the biology of learning and memory is the central involvement of the cAMP signal transduction pathway. In *Aplysia*, the cAMP second-messenger system is critically involved in neural events underlying both associative and non-associative modulation of a behavioral reflex (Kandel, E. R. and J. H. Schwartz, *Science*, 218: 433-443 (1982); Kandel, E. R., et al., *In Synaptic Function*, Edelman, G. M., et al. (Eds.), John Wiley and Sons, New York (1987); Byrne, J. H., et al., *In Advances in Second Messenger and Phosphoprotein Research*, Shenolikar, S. and A. C. Nairn (Eds.), Raven Press, New York, pp. 47-107 (1993)). In *Drosophila*, two mutants, *dunce* and *rutabaga*, were isolated in a behavioral screen for defects in associative learning and are lesioned in genes directly involved in cAMP metabolism (Quinn, W. G., et al., *Proc. Natl. Acad. Sci. USA*, 71: 708-712 (1974); Dudai, Y., et al., *Proc. Natl. Acad. Sci. USA* 73: 1684-1688 (1976); Byers, D. et al., *Nature*, 289: 79-81 (1981); Livingstone, M. S., et al., *Cell*, 37: 205-215 (1984); Chen, C. N. et al., *Proc. Natl. Acad. Sci. USA*, 83: 9313-9317 (1986); Levin, L. R., et al., *Cell*, 68: 479-489 (1992)). These latter observations were extended with a reverse-genetic approach using inducible transgenes expressing peptide inhibitors of cAMP-dependent protein kinase (PKA) and with analyses of mutants in the PKA catalytic subunit (Drain, P. et al., *Neuron*, 6: 71-82 (1991); Skoulakis, E. M., et al., *Neuron*, 11: 197-208 (1993)). Recent work on mammalian long-term potentiation (LTP) also has indicated a role for cAMP in synaptic plasticity (Frey, U., et al., *Science*, 260: 1661-1664 (1993); Huang, Y. Y. and E. R. Kandel, *In Learning and Memory*, vol. 1, pp. 74-82, Cold Spring Harbor Press, Cold Spring Harbor, N.Y. (1994)).

The formation of long-lasting memory in animals and of long-term facilitation in *Aplysia* can be disrupted by drugs that interfere with transcription or translation (Agranoff, B. W. et al., *Brain Res.*, 1: 303-309 (1966); Barondes, S. H. and H. D. Cohen, *Nature*, 218: 271-273 (1968); Davis, H. P. and L. R. Squire, *Psychol. Bull.*, 96: 518-559 (1984); Rosenzweig, M. R. and E. L. Bennett, *In Neurobiology of Learning and Memory*, Lynch, G., et al. (Eds.), The Guilford Press, New York, pp. 263-288, (1984); Montarolo, P. G., et al., *Science*, 234: 1249-1254 (1986)). This suggests that memory consolidation requires *de novo* gene expression. Considered along with the involvement of the cAMP second-messenger pathway, this requirement for newly synthesized gene products suggests a role for cAMP-dependent gene expression in long-term memory (LTM) formation.

In mammals, a subset of genes from the CREB/ATF family are known to mediate cAMP-responsive transcription (Habenner, J. F., *Mol. Endocrinol.*, 4: 1087-1094 (1990); deGroot, R. P. and P. Sassone-Corsi, *Mol. Endocrinol.*, 7: 145-153 (1993)). CREBs are members of the basic region-leucine zipper transcription factor superfamily; (Landschulz, W. H. et al., *Science*, 240: 1759-1764 (1988)). The leucine zipper domain mediates selective homo- and hetero-dimer formation among family members (Hai, T. Y. et al., *Genes & Dev.*, 3: 2083-2090 (1989); Hai, T. and T. Curran, *Proc. Natl. Acad. Sci. USA*, 88: 3720-3724 (1991)). CREB dimers bind to a conserved enhancer element (CRE) found in the upstream control region of many cAMP-responsive mammalian genes (Yamamoto, K. K., et al., *Nature*, 334: 494-498 (1988)). Some CREBs become transcriptional activators when spe-

cifically phosphorylated by PKA (Gonzalez, G. A. and M. R. Montminy, *Cell*, 59: 675-680 (1989); Foulkes, N. S. et al., *Nature*, 355: 80-84 (1992)), while others, isoforms from the CREM gene, are functional antagonists of these PKA-responsive activators (Foulkes, N. S. et al., *Cell*, 64: 739-749 (1991); Foulkes, N. and P. Sassone-Corsi, *Cell*, 68: 411-414 (1992)).

Work in *Aplysia* has shown that cAMP-responsive transcription is involved in long-term synaptic plasticity (Schacher, S. et al., *Science*, 240: 1667-1669 (1988); Dash, P. K., *Nature*, 345: 718-721 (1990)). A primary neuronal coculture system has been used to study facilitation of synaptic transmission between sensory and motor neurons comprising the monosynaptic component of the *Aplysia* gill-withdrawal reflex. Injection of oligonucleotides containing CRE sites into the nucleus of the sensory neuron specifically blocked long-term facilitation (Dash, P. K., *Nature*, 345: 718-721 (1990)). This result suggests that titration of CREB activity might disrupt long-term synaptic plasticity.

Described herein is the cloning and characterization of a *Drosophila* CREB gene, dCREB2. This gene produces several isoforms that share overall structural homology and nearly complete amino acid identity in the basic region-leucine zipper with mammalian CREBs. The dCREB2-a isoform is a PKA-responsive transcriptional activator whereas the dCREB2-b product blocks PKA-responsive transcription by dCREB2-a in cell culture. These molecules with opposing activities are similar in function to isoforms of the mammalian CREM gene (Foulkes, N. S. et al., *Cell*, 64: 739-749 (1991); Foulkes, N. and P. Sassone-Corsi, *Cell*, 68: 411-414 (1992); Foulkes, N. S. et al., *Nature*, 355: 80-84 (1992)). The numerous similarities in sequence and function between dCREB2 and mammalian CREBs suggest that cAMP-responsive transcription is evolutionarily conserved.

Genetic studies of memory formation in *Drosophila* have revealed that the formation of a protein synthesis-dependent long-term memory (LTM) requires multiple training sessions with a rest interval between them. As discussed further herein, this LTM is blocked specifically by induced expression of a repressor isoform of the cAMP-responsive transcription factor CREB. Also as discussed further herein, LTM information is enhanced after induced expression of an activator form of CREB. Maximum LTM is achieved after just one training session.

To investigate the role of CREBs in long-term memory (LTM) formation in *Drosophila*, dominant-negative transgenic lines which express dCREB2-b under the control of a heat-shock promoter (hs-dCREB2-b) were generated. Groups of flies, which had been heat-shock induced or left uninduced, were tested for memory retention after Pavlovian olfactory learning. This acute induction regimen minimized potential complications from inappropriate expression of dCREB2-b during development and allowed a clear assessment of the effect of hs-dCREB2-b induction on memory formation.

In *Drosophila*, consolidated memory after olfactory learning is composed of two genetically distinct components: anesthesia-resistant memory (ARM) and long-term memory (LTM). ARM decays to zero within four days after training, and formation of ARM is insensitive to the protein synthesis inhibitor cycloheximide (CXM) but is disrupted by the radish mutation (Folkers, E., et al., *Proc. Natl. Acad. Sci. USA*, 90: 8123-8127 (1993)). In contrast, LTM shows essentially no decay over at least seven days, its formation is cycloheximide-sensitive and it is not disrupted by radish. Two different training protocols involving massed and spaced sessions were employed (Ebbinghaus, H., *Über das Gedächtnis*, Dover,

N.Y.(1885); Baddeley, A. D., *The Psychology of Memory*, Basic Books, N.Y. (1976)) to dissect memory formation. The massed training procedure consists of ten consecutive training cycles with no rest interval between them, while the spaced training protocol consists of the same number of sessions but with a 15-minute rest between each. Their genetic dissection revealed that the massed protocol produced only ARM, while the spaced protocol produced memory retention composed of both ARM and LTM.

The behavioral results show that formation of LTM is completely blocked by induced expression of hs-dCREB2-b. This effect is remarkable in its behavioral specificity. ARM, a form of consolidated memory genetically distinguishable from LTM, but co-existing with it one-day after spaced training, was not affected. Learning and peripheral behaviors likewise were normal. Thus, the effect of the induced hs-dCREB2-b transgene is specific to LTM.

Induction of the mutant blocker did not affect LTM. This result, together with the molecular data which showed that induction of the wild-type blocker did not have widespread effects on transcription, suggests that the blocker is reasonably specific at the molecular level when it specifically blocks LTM. The wild-type blocker may disrupt cAMP-dependent transcription in vivo, since it can block cAMP-responsive transcription in cell culture. It is reasonable to infer that dimerization is necessary for blocker function and that the wild-type blocker could interfere with cAMP-responsive transcription either by forming heterodimers with dCREB2-a, the activator, or by forming homodimers and competing for DNA binding with homodimers of dCREB2-a. Thus, activators and repressors may form homodimers or heterodimers. It is reasonable to infer that long term memory is enhanced when the level of activator homodimer is increased from normal and/or when the level of activator-repressor heterodimer is decreased from normal and/or when the level of repressor homodimer is decreased from normal. In any case, the molecular target(s) of dCREB2-b are likely to be interesting because of the behavioral specificity of the block of LTM.

In *Drosophila*, consolidation of memory into long-lasting forms is subject to disruption by various agents. A single-gene mutation *radish* and the pharmacological agent CXM were used to show that long-lasting memory in flies is dissectable into two components, a CXM-insensitive ARM, which is disrupted by *radish*, and a CXM-sensitive LTM, which is normal in *radish* mutants. As described herein, CREB-family members are likely to be involved in the CXM-sensitive, LTM branch of memory consolidation. The results described herein, taken together with the showing that long-term memory is dissectable into a CXM-insensitive ARM and a CXM-sensitive LTM, show that only one functional component of consolidated memory after olfactory learning lasts longer than four days, requires *de novo* protein synthesis and involves CREB-family members.

Based on work in *Aplysia*, a model has been proposed to describe the molecular mechanism(s) underlying the transition from short-term, protein synthesis-independent to long-term, protein synthesis-dependent synaptic plasticity (Alberini, C. M. et al., *Cell*, 76: 1099-1114 (1994)). The present work in *Drosophila* on long-term memory extends this model to the whole organism. Important molecular aspects of this transition seem to involve migration of the catalytic subunit of PKA into the nucleus (Backsai, B. J. et al., *Science*, 260: 222-226 (1993)) and subsequent phosphorylation and activation of CREB-family members (Dash, P. K., *Nature*, 345: 718-721 (1990); Kaang, B. K., et al., *Neuron*, 10: 427-435 (1993)). In flies, it is likely that the endogenous dCREB2-a isoform is one of these nuclear targets. Activated dCREB2-a

molecules then might transcribe other target genes, including the immediate early genes—as is apparently the case in *Aplysia*. (Alberini, C. M. et al., *Cell*, 76: 1099-1114 (1994)).

It is remarkable that the cAMP signal transduction pathway, including its nuclear components, seem to be required for memory-related functions in each of these species and behavioral tasks. Taken together with cellular analyses of a long-lasting form of LTP in hippocampal slices (Frey, U., et al., *Science*, 260: 1661-1664 (1993); Huang, Y. Y. and E. R. Kandel, In *Learning and Memory*, vol. 1, pp. 74-82, Cold Spring Harbor Press, Cold Spring Harbor, N.Y. (1994)), the emerging picture is that cAMP-responsive transcription is a conserved molecular switch involved in the consolidation of short-term memory to long-term memory. Thus, it is reasonable to infer that differential regulation of CREB isoforms serves as a molecular switch for the formation of long term memory.

A universal property of memory formation is that spaced training (repeated training sessions with a rest interval between them) produces stronger, longer-lasting memory than massed training (the same number of training sessions with no rest interval) (Ebbinghaus, H., *Über das Gedächtnis*, Dover, N.Y. (1885); Hintzman, D. L., In *Theories in Cognitive Psychology: The Loyola Symposium*, R. L. Solso (Ed.), pp. 77-99, Lawrence Erlbaum Assoc., Hillsdale, N.J. (1974); Carew, T. J., et al., *Science*, 175: 451-454 (1972); Frost, W. N., et al., *Proc. Natl. Acad. Sci. USA*, 82: 8266-8269 (1985)). This phenomenon also exists in fruit flies for a conditioned odor avoidance response (Tully, T. and W. G. Quinn, *J. Comp. Physiol.* 157: 263-277 (1985)). Genetic dissection of this long-lasting memory has revealed, however, an important difference between massed and spaced training. Spaced training produces two functionally independent forms of consolidated memory, ARM and LTM, while massed training produces only ARM.

As described herein, ARM and LTM differ primarily in their requirement for protein synthesis during induction. ARM is not affected when flies are fed the protein synthesis inhibitor cycloheximide (CXM) immediately before or after training, while LTM is completely blocked under the same feeding conditions. ARM in normal flies also decays away within four days after training, while LTM shows no decay for at least seven days. Thus, protein synthesis is required to induce LTM, but LTM is maintained indefinitely once formed. These latter properties of LTM have been observed throughout the animal kingdom (Davis, H. P. and L. R. Squire, *Psychol. Bull.*, 96: 518-559 (1984); Castellucci, V. F., et al., *J. Neurobiol.*, 20: 1-9 (1989); Erber, J., *J. Comp. Physiol. Psychol.*, 90: 41-46 (1976); Jaffe, K., *Physiol. Behav.*, 25: 367-371 (1980)). The emerging neurobiological interpretation is that formation of LTM involves protein synthesis-dependent structural changes at relevant synapses (Greenough, W. T., *TINS*, 7: 229-283 (1984); Buonomano, D. V. and J. H. Byrne, *Science*, 249: 420-423 (1990); Nazif, F. A., et al., *Brain Res.*, 539: 324-327 (1991); Stewart, M. G., In *Neural and Behavioural Plasticity: The Use of the Domestic Chick As A Model*, R. J. Andrew (Ed.), pp. 305-328, Oxford, Oxford (1991); Bailey, C. H. and E. R. Kandel, *Sem. Neurosci.*, 6:35-44 (1994)). The modern molecular view is that regulation of gene expression underlies this protein synthesis-dependent effect (Golet, P. et al., *Nature*, 322: 419-422 (1986); Gall, C. M. and J. C. Lauterborn, In *Memory: Organization and Locus of Change*, L. R. Squire, et al., (Eds.) pp. 301-329 (1991); Armstrong, R. C. and M. R. Montminy, *Annu. Rev. Neurosci.*, 16: 17-29 (1993)).

Why is spaced training required to induce LTM? The massed and spaced procedures both entail ten training ses-



sions; consequently, flies receive equivalent exposure to the relevant stimuli (one odor temporally paired with electric shock and a second odor presented without shock). The only procedural difference between massed and spaced training is the rest interval between each training session. The absence of a rest interval between sessions during massed training does not appear to disrupt the memory formation process. The level of initial learning assayed immediately after massed training is similar to that after spaced training. In addition, ARM levels are similar after both training procedures. Thus, the presence of a rest interval during spaced training seems crucial to the induction of LTM.

To investigate the temporal kinetics of this rest interval in relation to the formation of LTM (FIGS. 13A and 13B), it was first established that the usual ten sessions of spaced training produced maximal 7-day memory retention (7-day retention is composed solely of LTM, since ARM decays to zero within four days).

FIG. 13A shows that 15 or 20 training sessions did not improve memory retention. Thus, ten spaced training sessions produces maximal, asymptotic levels of LTM.

LTM as a function of the length of the rest interval between 10 spaced training sessions was then assessed. FIG. 13B reveals a continuous increase in LTM from a 0-min rest interval (massed training) to a 10-minute rest interval, at which time LTM levels reach maximum. Longer rest intervals yielded similar memory scores. These observations of LTM formation suggest an underlying biological process, which changes quantitatively during the rest interval between sessions and which accumulates over repeated training sessions.

In transgenic flies, the formation of LTM, but not ARM or any other aspect of learning or memory, is disrupted by induced expression of a repressor form of the cAMP-responsive transcription factor CREB (Example 4). Mutating two amino acids in the “leucine zipper” dimerization domain of this CREB repressor was sufficient to prevent the dominant-negative effect on LTM. Thus, induction of LTM is not only protein synthesis-dependent but also is CREB-dependent. Stated more generally, CREB function is involved specifically in a form of a memory that is induced only by spaced training. This observation was particularly intriguing in light of the molecular nature of CREB.

In *Drosophila*, transcriptional and/or post-translational regulation of dCREB2 yields several mRNA isoforms. Transient transfection assays in mammalian F9 cells have demonstrated that one of these isoforms (CREB2-a) functions as a cAMP-responsive activator of transcription, while a second isoform (CREB2-b) acts as an antagonistic repressor of the activator (Example 1; cf. Habener, J. F., *Mol. Endocrinol.*, 4: 1087-1094 (1990); Foulkes, N. and P. Sassone-Corsi, *Cell*, 68: 411-414 (1992)). (This repressor isoform was used previously to generate the inducible transgene mentioned above.) The existence of different CREB isoforms with opposing functions suggested an explanation for the requirement of multiple training sessions with a rest interval between them for the formation of LTM.

In its simplest form, this model (Example 7; FIG. 14) supposes that cAMP-dependent protein kinase (PKA), activated during training, induces the synthesis and/or function of both CREB activator and repressor isoforms (cf. Yamamoto, K. K., et al., *Nature*, 334: 494-498 (1988); Backsai, B. J. et al., *Science*, 260: 222-226 (1993)). Immediately after training, enough CREB repressor exists to block the ability of CREB activator to induce downstream events. Then, CREB repressor isoforms are inactivated faster than CREB activator isoforms. In this manner, the net amount of functional activator ( $\Delta C = \text{CREB2a} - \text{CREB2b}$ ) increases during a rest interval and

then accumulates over repeated training sessions (with a rest interval) to induce further the downstream targets involved with the formation of LTM (Montarolo, P. G., et al., *Science*, 234: 1249-1254 (1986); Kaang, B. K., et al., *Neuron*, 10: 427-435 (1993)).

This model leads to three predictions, which have been confirmed. First, if the functional difference between CREB activator and repressor isoforms is zero ( $\Delta C = 0$ ) immediately after one training session, then additional massed training sessions should never yield LTM. FIG. 15A shows that 48 massed training sessions, rather than the usual 10, still does not produce any 7-day memory retention. Second, if the amount of CREB repressor is increased experimentally,  $\Delta C$  will be negative immediately after training ( $\Delta C < 0$ ). Then, enough CREB repressor may not decay during a rest interval to free enough CREB activator for induction of LTM. This has been shown to be the case for spaced training (15-min rest interval) after inducing the expression of a hsp-dCREB2-b (repressor) transgene three hours before training (Example 4). Third, if the amount of CREB activator is increased experimentally,  $\Delta C$  will be positive immediately after training ( $\Delta C > 0$ ). This effect, then, should eliminate or reduce the rest interval required to induce LTM. FIG. 15B shows the results from recent experiments in which the expression of a hsp-dCREB2-a (activator) transgene was induced three hours before training. In these transgenic flies, massed training produced maximal LTM. This effect appeared not to arise trivially, since olfactory acuity, shock reactivity (FIG. 15C) and initial learning were normal in transgenic flies after heat shock-induction. Thus, the requirement for a rest interval between training sessions to induce LTM specifically was eliminated.

FIG. 15B also shows that maximal LTM occurred in induced hsp-dCREB2-a transgenic flies after just one training session. The usual requirement for additional training to form a strong, long-lasting memory was no longer necessary. Thus, induced overexpression of a CREB activator has produced in otherwise normal flies, the functional equivalent of a “photographic” memory. This result indicates that the amount of CREB activator present during training—rather than the amount of activated PKA that reaches CREB in the nucleus, for instance (cf. Backsai, B. J. et al., *Science*, 260: 222-226 (1993); Kaang, B. K., et al., *Neuron*, 10: 427-435 (1993); Frank, D. A. and M. E. Greenberg, *Cell*, 79: 5-8 (1994))—is the rate-limiting step of LTM formation. Taken together, results from these experiments support the notion that the opposing functions of CREB activators and repressors act as a “molecular switch” (cf. Foulkes, N. S. et al., *Nature*, 355: 80-84 (1992)) to determine the parameters of extended training (number of training sessions and rest interval between them) required to form maximum LTM.

To date, seven different dCREB2 RNA isoforms have been identified, and more are hypothesized to exist. Each may be regulated differentially at transcriptional (Meyer, T. E., et al., *Endocrinology*, 132: 770-780 (1993)) and/or translation levels before or during LTM formation. In addition, different combinations of CREB isoforms may exist in different (neuronal) cell types. Consequently, many different combinations of activator and repressor molecules are possible. From this perspective, the notions that all activators and repressors are induced during a training session or that all repressors inactivate faster than activators (see above) need not be true. Instead, the model requires only that  $\Delta C$  (the net function of activators and repressors) is less than or equal to zero immediately after training and increases with time (rest interval).

Theoretically, particular combinations of activator and repressor molecules in the relevant neuron(s) should deter-

mine the rest interval and/or number of training sessions necessary to produce maximum LTM for any particular task or species. Thus, the molecular identification and biochemical characterization of each CREB activator and repressor isoform used during LTM formation in fruit flies is the next major step toward establishing the validity of our proposed model. Similar experiments in other species may establish its generality.

CREB certainly is not involved exclusively with LTM. The dCREB2 gene, for instance, is expressed in all fruit fly cells and probably acts to regulate several cellular events (Foulkes, N. S. et al., *Nature*, 355: 80-84 (1992)).

So, what defines the specificity of its effects on LTM? Specificity most likely resides with the neuronal circuitry involved with a particular learning task. For olfactory learning in fruit flies, for instance, CREB probably is modulated via the cAMP second messenger pathway. Genetic disruptions of other components of this pathway are known to affect olfactory learning and memory (Livingstone, M. S., et al., *Cell*, 37: 205-215 (1984); Drain, P. et al., *Neuron*, 6: 71-82 (1991); Levin, L. R., et al., *Cell*, 68: 479-489 (1992); Skoulakis, E. M., et al., *Neuron* 11: 197-208 (1993); Qiu, Y. and R. L. Davis, *Genes Develop.* 7: 1447-1458 (1993)). Presumably, the stimuli used during conditioning (training) stimulate the underlying neuronal circuits. The cAMP pathway is activated in (some) neurons participating in the circuit, and CREB-dependent regulation of gene expression ensues in the "memory cells". This neurobiological perspective potentially will be established in *Drosophila* by identifying the neurons in which LTM-specific CREB function resides. Experiments using a neuronal co-culture system in *Aplysia* already have contributed significantly to this issue (Alberini, C. M. et al., *Cell*, 76: 1099-1114 (1994) and references therein).

The involvement of CREB in memory, or in the structural changes of neurons which underlie memory in vivo, also has been implicated in mollusks (Dash, P. K., *Nature*, 345: 718-721 (1990); Alberini, C. M. et al., *Cell*, 76: 1099-1114 (1994)) and in mice (Bourtchuladze, R., et al., *Cell*, 79: 59-68 (1994)). Ample evidence also exists for the involvement of the cAMP second messenger pathway in associative learning in *Aplysia* (Kandel, E. R., et al., *In Synaptic Function*, Edelman, G. M., et al. (Eds.), John Wiley and Sons, New York (1987); Byrne, J. H., et al., *In Advances in Second Messenger and Phosphoprotein Research*, Shenolikar, S. and A. C. Nairn (Eds.), Raven Press, New York, pp. 47-107 (1993)) and in rat hippocampal long-term potentiation (LTP), a cellular model of associative learning in vertebrates (Frey, U., et al., *Science*, 260: 1661-1664 (1993); Huang, Y. Y. and E. R. Kandel, *In Learning and Memory*, vol. 1, pp. 74-82, Cold Spring Harbor Press, Cold Spring Harbor, N.Y. (1994)). Finally, cellular and biochemical experiments have suggested that CREB function may be modulated by other second messenger pathways (Dash, P. K., et al., *Proc. Natl. Acad. Sci. USA* 88: 5061-5065 (1991); Ginty, D. D. et al., *Science*, 260: 238-241 (1993); deGroot, R. P. and P. Sassone-Corsi, *Mol. Endocrinol.*, 7: 145-153 (1993)). These observations suggest that CREB might act as a molecular switch for LTM in many species and tasks.

Finally, why might the formation of LTM require a molecular switch? Many associative events occur only once in an animal's lifetime. Forming long-term memories of such events would be unnecessary and if not counterproductive. Instead, discrete events experienced repeatedly are worth remembering. In essence, a recurring event comprises a relevant signal above the noise of one-time events. Teleologically, then, the molecular switch may act as an information filter to ensure that only discrete but recurring events are

remembered. Such a mechanism would serve efficiently to tailor an individual's behavioral repertoire to its unique environment.

The present invention also relates to isolated DNA having sequences which encode (1) a cyclic 3',5'-adenosine monophosphate (cAMP) responsive transcriptional activator, or a functional fragment thereof, or (2) an antagonist of a cAMP responsive transcriptional activator, or a functional fragment thereof, or (3) both an activator and an antagonist, or functional fragments thereof of both.

The invention relates to isolated DNA having sequences which encode *Drosophila* dCREB2 isoforms, or functional analogues of a dCREB2 isoform. As referred to herein, a functional analogue of a dCREB2 isoform comprises at least one function characteristic of a *Drosophila* dCREB2 isoform, such as a cAMP-responsive transcriptional activator function and/or an antagonistic repressor of the cAMP activator function. These functions (i.e., the capacity to mediate PKA-responsive transcription) may be detected by standard assays (e.g., assays which monitor for CREB-dependent activation). For example, assays in F9 cells have been used extensively to study CREB-dependent activation because their endogenous cAMP-responsive system is inactive; (Gonzalez, G. A. et al., *Nature*, 337: 749-752 (1989); Masson, N. et al., *Mol. Cell Biol.*, 12: 1096-1106 (1992); Masson, N. et al., *Nucleic Acids Res.*, 21: 1163-1169 (1993)).

The invention further relates to isolated DNA having sequences which encode a *Drosophila* dCREB2 gene or a functional fragment thereof. Isolated DNA meeting these criteria comprise nucleic acids having sequences identical to sequences of naturally occurring *Drosophila* dCREB2 and portions thereof, or variants of the naturally occurring sequences. Such variants include mutants differing by the addition, deletion or substitution of one or more nucleic acids.

The invention relates to isolated DNA that are characterized by (1) their ability to hybridize to a nucleic acid having the DNA sequence in FIG. 1A (SEQ ID NO.: 1) or its complement, or (2) by their ability to encode a polypeptide of the amino acid sequence in FIG. 1A (SEQ ID NO.: 2) or functional equivalents thereof (i.e., a polypeptide which functions as a cAMP responsive transcriptional activator), or (3) by both characteristics. Isolated nucleic acids meeting these criteria comprise nucleic acids having sequences homologous to sequences of mammalian CREB, CREM and ATF-1 gene products. Isolated nucleic acids meeting these criteria also comprise nucleic acids having sequences identical to sequences of naturally occurring dCREB2 or portions thereof, or variants of the naturally occurring sequences. Such variants include mutants differing by the addition, deletion or substitution of one or more residues, modified nucleic acids in which one or more residues is modified (e.g., DNA or RNA analogs), and mutants comprising one or more modified residues.

Such nucleic acids can be detected and isolated under high stringency conditions or moderate stringency conditions, for example. "High stringency conditions" and "moderate stringency conditions" for nucleic acid hybridizations are explained on pages 2.10.1-2.10.16 (see particularly 2.10.8-11) and pages 6.3.1-6 in *Current Protocols in Molecular Biology* (Ausubel, F. M. et al., eds, Vol. 1, Suppl. 26, 1991), the teachings of which are incorporated herein by reference. Factors such as probe length, base composition, percent mismatch between the hybridizing sequences, temperature and ionic strength influence the stability of nucleic acid hybrids. Thus, high or moderate stringency conditions can be determined empirically, depending in part upon the characteristics

of the known DNA to which other unknown nucleic acids are being compared for homology.

Isolated nucleic acids that are characterized by their ability to hybridize to a nucleic acid having the sequence in FIG. 1A or its complement (e.g., under high or moderate stringency conditions) may further encode a protein or polypeptide which functions as a cAMP responsive transcriptional activator.

The present invention also relates to isolated DNA having sequences which encode an enhancer-specific activator, or a functional fragment thereof.

The invention further relates to isolated DNA having sequences which encode a *Drosophila* dCREB 1 gene or a functional fragment thereof. Isolated DNA meeting these criteria comprise nucleic acids having sequences identical to sequences of naturally occurring *Drosophila* dCREB1 and portions thereof, or variants of the naturally occurring sequences. Such variants include mutants differing by the addition, deletion or substitution of one or more nucleic acids.

The invention further relates to isolated DNA that are characterized by (1) their ability to hybridize to a nucleic acid having the DNA sequence in FIG. 5 (SEQ ID NO.: 7) or its complement, or (2) by their ability to encode a polypeptide of the amino acid sequence in FIG. 5 (SEQ ID NO.: 8), or by both characteristics. Isolated DNA meeting these criteria also comprise nucleic acids having sequences identical to sequences of naturally occurring dCREB1 or portions thereof, or variants of the naturally occurring sequences. Such variants include mutants differing by the addition, deletion or substitution of one or more residues, modified nucleic acids in which one or more residues is modified (e.g., DNA or RNA analogs), and mutants comprising one or more modified residues.

Such nucleic acids can be detected and isolated under high stringency conditions or moderate stringency conditions as described above, for example.

Fragments of the isolated DNA which code for polypeptides having a certain function can be identified and isolated by, for example, the method of Jasin, M., et al., U.S. Pat. No. 4,952,501.

Nitric Oxide in Invertebrates: *Drosophila* dNOS Gene Codes for a Ca<sup>2+</sup>/Calmodulin-Dependent Nitric Oxide Synthase.

Nitric oxide (NO) is a gaseous mediator of a wide variety of biological processes in mammalian organisms. Applicants have cloned the *Drosophila* gene, dNOS, coding for a Ca<sup>2+</sup>/calmodulin-dependent nitric oxide synthase (NOS). Presence of a functional NOS gene in *Drosophila* provides conclusive evidence that invertebrates synthesize NO and presumably use it as a messenger molecule. Furthermore, conservation of an alternative RNA splicing pattern between dNOS and vertebrate neuronal NOS, suggests broader functional homology in biochemistry and/or regulation of NOS.

NO is synthesized by nitric oxide synthases (NOSs) during conversion of L-arginine to L-citrulline (Knowels, R. G., et al., *Biochem. J.*, 298: 249 (1994); Nathan, C., et al., *J. Biol. Chem.*, 269: 13725 (1994); Marletta, M. A., *J. Biol. Chem.*, 268: 12231 (1993)). Biochemical characterization of NOSs has distinguished two general classes: (i) constitutive, dependent on exogenous Ca<sup>2+</sup> and calmodulin and (ii) inducible, independent of exogenous Ca<sup>2+</sup> and calmodulin. Analyses of cDNA clones have identified at least three distinct NOS genes in mammals (Bredt, D. S., et al., *Nature*, 351: 714-718 (1991); Lamas, S., et al., *Proc. Natl. Acad. Sci. USA*, 89: 6348-6352 (1992); Lyons, C. R., et al., *J. Biol. Chem.*, 267: 6370 (1992); Lowenstein, C. J., et al., *Proc. Natl. Acad. Sci. USA*, 89: 6711 (1992); Sessa, W. C., et al., *J. Biol. Chem.*, 267:15274 (1992);

Geller, D. A., et al., *Proc. Natl. Acad. Sci. USA*, 90: 3491 (1993); Xie, Q. et al., *Science*, 256: 225-228 (1992)) neuronal, endothelial and macrophage, the former two of which are constitutive and the latter of which is inducible. The nomenclature for these different isoforms used here is historical, as it is clear now that one or more isoforms can be present in the same tissues (Dinerman, J. L., et al., *Proc. Natl. Acad. Sci. USA*, 91: 4214-4218 (1994)).

As a diffusible free-radical gas, NO is a multifunctional messenger affecting many aspects of mammalian physiology [for reviews, see Dawson, T. M., et al., *Ann. Neurol.*, 32: 297 (1992); Nathan, C., *FASEB J.*, 6: 3051 (1992); Moncada, S., et al., *N. Eng. J. Med.*, 329: 2002-2012 (1993); Michel, T., et al., *Amer. J. Cardiol.*, 72: 33C (1993); Schuman, E. M., et al., *Annu. Rev. Neurosci.*, 17: 153-183 (1994)]. NO originally was identified as an endothelium-derived relaxing factor responsible for regulation of vascular tone (Palmer, R. M. J., *Nature*, 327: 524 (1987); Palmer, R. M. J., et al., *Nature*, 333: 664 (1988); Ignarro, L. J., et al., *Proc. Natl. Acad. Sci. USA*, 84: 9265 (1987)) and as a factor involved with macrophage-mediated cytotoxicity (Marletta, M. A., et al., *Biochemistry*, 21: 8706 (1988); Hibbs, J. B., et al., *Biochem. Biophys. Res. Comm.*, 157: 87 (1989); Steuhr, D. J., et al., *J. Exp. Med.*, 169: 1543 (1989)). Since NO has been implicated in several physiological processes including inhibition of platelet aggregation, promotion of inflammation, inhibition of lymphocyte proliferation and regulation of microcirculation in kidney (Radomski, M., et al., *Proc. Natl. Acad. Sci. USA*, 87: 5193 (1990); Albina, J. E., *J. Immunol.*, 147: 144 (1991); Katz, R., *Am. J. Physiol.*, 261: F360 (1992); Ialenti, A., et al., *Eur. J. Pharmacol.*, 211: 177 (1992)). More recently, NO also has been shown to play a role in cell-cell interactions in mammalian central and peripheral nervous systems—in regulating neurotransmitter release, modulation of NMDA receptor-channel functions, neurotoxicity, nonadrenergic noncholinergic intestinal relaxation (Uemura, Y., et al., *Ann. Neurol.*, 27: 620-625 (1990)) and activity-dependent regulation of neuronal gene expression (Uemura, Y., et al., *Ann. Neurol.*, 27: 620 (1990); Dawson, V. L., et al., *Proc. Natl. Acad. Sci. USA*, 88: 6368 (1991); Lei, S. Z., et al., *Neuron*, 8: 1087 (1992); Prast, H., et al., *Eur. J. Pharmacol.*, 216: 139 (1992); Peunova, N., *Nature*, 364: 450 (1993)). Recent reports of NO function in synaptogenesis and in apoptosis during development of the rat CNS (Bredt, D. S., *Neuron*, 13: 301 (1994); Roskams, A. J., *Neuron*, 13: 289 (1994)) suggest that NO regulates activity-dependent mechanism(s) underlying the organization of fine-structure in the cortex (Edelman, G. M., et al., *Proc. Natl. Acad. Sci. USA*, 89: 11651-11652 (1992)). NO also appears to be involved with long-term potentiation in hippocampus and long-term depression in cerebellum, two forms of synaptic plasticity that may underlie behavioral plasticity (Bohme, G. A., *Eur. J. Pharmacol.*, 199: 379 (1991); Schuman, E. M., *Science* 254: 1503 (1991); O'Dell, T. J., et al., *Proc. Natl. Acad. Sci. USA*, 88: 11285 (1991); Shibuki, K., *Nature*, 349: 326 (1991); Haley, J. E., et al., *Neuron*, 8: 211 (1992); Zhuo, M., *Science*, 260: 1946 (1993); Zhuo, M., et al., *Neuro Report*, 5: 1033 (1994)). Consistent with these cellular studies, inhibition of NOS activity has been shown to disrupt learning and memory (Chapman, P. F., et al., *Neuro Report*, 3: 567 (1992); Holscher, C., *Neurosci. Lett.*, 145: 165 (1992); Bohme, G. A., et al., *Proc. Natl. Acad. Sci. USA*, 90: 9191 (1993); Rickard, N. S., *Behav. Neurosci.*, 108: 640-644 (1994)).

Many of the above conclusions are based on pharmacological studies using inhibitors of nitric oxide synthases or donors of NO. Interpretations of such studies usually are limited because the drugs interact with more than one target

and they cannot be delivered to specific sites. A molecular genetic approach can overcome these problems, however, by disrupting a specific gene, the product of which may be one of the drug's targets. Recently, such an approach has been attempted in mice via generation of a knock-out mutation of the neuronal NOS (nNOS) (Huang, P. L., et al., *Cell*, 75: 1273-1286 (1993)). While nNOS mutants appeared fully viable and fertile, minor defects in stomach morphology and hippocampal long-term potentiation were detected (Huang, P. L., et al., *Cell*, 75: 1273-1286 (1993); O'Dell, T. J., et al., *Science*, 265: 542-546 (1994)). Moreover, some NOS enzymatic activity still was present in certain regions of the brain, suggesting a role for other NOS genes in the CNS. While yielding some relevant information about one specific component of NO function, this nNOS disruption existed throughout development. Consequently, functional defects of NOS disruption in adults could not be resolved adequately from structural defects arising during development. Genetic tools exist in *Drosophila*, in contrast, to limit disruptions of gene functions temporally or spatially.

To identify candidate *Drosophila* NOS homologs, a fragment of the rat neuronal NOS cDNA (Bredt, D. S., et al., *Nature*, 351: 714-718 (1991)) was hybridized at low stringency to a phage library of the *Drosophila* genome as described in Example 11. The rat cDNA fragment encoded the binding domains of FAD and NADPH (amino acids 979-1408 of SEQ ID NO.: 11), which are cofactors required for NOS activity, and therefore were expected to be conserved in fruit flies. Several *Drosophila* genomic clones were identified with the rat probe and classified into eight contigs. Sequence analysis of three restriction fragments from these genomic clones revealed one (2.4R) with high homology to mammalian NOSs. The deduced amino acid sequence of the ORF encoded within the 2.4R fragment indicated 40% identity to the rat neuronal NOS and binding sites for FAD and NADPH.

The 2.4R DNA fragment then was used to probe a *Drosophila* adult head cDNA library as described in Example 11, and eight clones were isolated. Restriction analysis indicated that all contained identical inserts and thus, defined a predominant transcript expressed by this *Drosophila* gene. One clone (c5.3) was sequenced in both directions. The 4491 bp cDNA contained one long ORF of 4350 bp. The methionine initiating this ORF was preceded by ACAAG which is a good match to the translation start consensus (A/CAAA/C) for *Drosophila* genes (Cavener, D. R., *Nucleic Acids Res.*, 15: 1353-1361 (1987)). Conceptual translation of this ORF yielded a protein of 1350 amino acids with a molecular weight of 151,842 Da.

Comparison of the amino acid sequence of this deduced *Drosophila* protein (DNOS) (SEQ ID NO.: 9) to sequences of mammalian NOSs revealed that DNOS is 43% identical to neuronal NOS (SEQ ID NO.: 11), 40% identical to endothelial NOS (SEQ ID NO.: 10) and 39% identical to macrophage NOS (SEQ ID NO.: 12). It also revealed similar structural motifs in DNOS (FIG. 16A-16C). The C-terminal half of the DNOS protein contains regions of high homology corresponding to the presumptive FMN-, FAD- and NADPH-binding sites. Amino acids thought to be important for making contacts with FAD and NADPH in mammalian NOSs (Bredt, D. S., et al., *Nature*, 351: 714-718 (1991); Lamas, S., et al., *Proc. Natl. Acad. Sci. USA*, 89: 6348-6352 (1992); Lyons, C. R., et al., *J. Biol. Chem.*, 267: 6370 (1992); Lowenstein, C. J., et al., *Proc. Natl. Acad. Sci. USA*, 89: 6711 (1992); Sessa, W. C., et al., *J. Biol. Chem.*, 267: 15274 (1992); Geller, D. A., et al., *Proc. Natl. Acad. Sci. USA*, 90: 3491 (1993); Xie, Q. et al., *Science*, 256: 225-228 (1992)) are conserved in DNOS. The middle section of DNOS, between residues 215 and 746 of

SEQ ID NO.: 9, showed the highest similarity to mammalian NOSs: it is 61% identical to the neuronal isoform and 53% identical to endothelial and macrophage isoforms. Sequences corresponding to the proposed heme- and calmodulin-binding sites in mammalian enzymes are well-conserved in DNOS. The region located between residues 643-671 of SEQ ID NO.: 9 has the characteristics of a calmodulin-binding domain (basic, amphiphilic  $\alpha$ -helix) (O'Neil, K. T., et al., *Trends Biochem. Sci.*, 15: 59-64 (1990)). The amino acid sequence between these two sites is very well conserved among all four NOS proteins, suggesting the location of functionally important domains such as the arginine-binding site (Lamas, S., et al., *Proc. Natl. Acad. Sci. USA*, 89: 6348-6352 (1992)), tetrahydrobiopterine cofactor binding site or a dimerization domain. DNOS also has a PKA consensus site (Pearson, R. B., *Meth. Enzymol.*, 200: 62-81 (1991)) (at Ser-287 of SEQ ID NO.: 9) in a position similar to neuronal and endothelial NOSs.

The 214 amino acid N-terminal domain of DNOS shows no obvious homology to its equivalent portion of neuronal NOS or to the much shorter N-terminal domains of endothelial and macrophage NOSs. This region of DNOS contains an almost uninterrupted homopolymeric stretch of 24 glutamine residues. Such glutamine-rich domains, found in many *Drosophila* and vertebrate proteins, have been implicated in protein-protein interactions regulating the activation of transcription (Franks, R. G., *Mech. Dev.*, 45: 269 (1994); Gerber, H.-P., et al., *Science*, 263: 808 (1994); Regulski, M., et al., *EMBO J*, 6: 767 (1987)). Thus, this domain of DNOS could be involved with protein-protein interactions necessary for localization and/or regulation of DNOS activity.

The above sequence comparisons suggest that a *Drosophila* structural homolog of a vertebrate NOS gene was identified. The order of the putative functional domains in the DNOS protein is identical to that of mammalian enzymes (FIG. 15B). Structural predictions based on several protein algorithms also indicate that general aspects of DNOS protein secondary structure (hydrophobicity plot, distribution of  $\alpha$ -helices and  $\beta$ -strands) from the putative heme-binding domain to the C-terminus are similar to those of mammalian NOSs. DNOS also does not contain a transmembrane domain, as is the case for vertebrate NOSs. In addition to these general characteristics, several aspects of DNOS structure actually render it most like neuronal NOS: (i) the overall sequence similarity, (ii) the similarity of the putative calmodulin-binding site (55% identical to the neuronal NOS vs. 45% identical to endothelial NOS or vs. 27% identical to macrophage NOS) and (iii) the large N-terminal domain. Neuronal NOS and DNOS also do not contain sites for N-terminal myristoylation, which is the case for endothelial NOS (Lamas, S., et al., *Proc. Natl. Acad. Sci. USA*, 89: 6348-6352 (1992)), nor do they have a deletion in the middle of the protein, which is the case for macrophage NOS (Xie, Q. et al., *Science*, 256: 225-228 (1992)).

To establish that Applicants putative DNOS protein had nitric oxide synthase activity, the dNOS cDNA was expressed in 293 human embryonic kidney cells as described in Example 12, which have been used routinely in studies of mammalian NOSs (Bredt, D. S., et al., *Nature*, 351: 714-718 (1991)). Protein extracts prepared from dNOS-transfected 293 cells as described in Example 12, contained a 150 kD polypeptide, which was recognized by a polyclonal antibody raised against the N-terminal domain of DNOS (FIG. 17A, lane 293+dNOS). This immunoreactive polypeptide was of a size expected for DNOS and was absent from cells transfected with just the pCGN vector alone (FIG. 17A, lane 293+vector).

Extracts made from dNOS-transfected 293 cells showed significant NO synthase activity, as measured by the L-arginine to L-citrulline conversion assay as described in Example 12 ( $0.1276 \pm 0.002$  pmol/mg/min; FIG. 17B, group B). [In a parallel experiment, the specific activity of rat neuronal NOS expressed from the same vector in 293 cells was  $3.0 \pm 0.02$  pmol/mg/min, N=4]. DNOS activity was dependent on exogenous  $Ca^{2+}$ /calmodulin and on NADPH, two cofactors necessary for activity of constitutive mammalian NOSs (Iyengar, R., *Proc. Natl. Acad. Sci. USA*, 84: 6369-6373 (1987); Bredt, D. S., *Proc. Natl. Acad. Sci. USA*, 87: 682-685 (1990)). DNOS activity was reduced 90% by the  $Ca^{2+}$  chelator EGTA (FIG. 17B, group C). Also, 500  $\mu$ M N-(6-aminohexyl)-1-naphthalene-sulfonamide (W5), a calmodulin antagonist which inhibits activity of neuronal NOS (Bredt, D. S., *Proc. Natl. Acad. Sci. USA*, 87: 682-685 (1990)), diminished DNOS activity to 18% ( $0.0222 \pm 0.001$  pmol/mg/min, N=2). In the absence of exogenous NADPH, DNOS (or nNOS) activity was reduced 20% ( $0.1061 \pm 0.011$  pmol/mg/min, N=4 for DNOS;  $2.7935 \pm 0.033$  pmol/mg/min, N=2 for nNOS). DNOS activity also was blocked by inhibitors of mammalian NOSs (Rees, D. D., *Br. J. Pharmacol.*, 101: 746-752 (1990)).  $N^G$ -nitro-L-arginine methyl ester (L-NAME) reduced DNOS activity 84% (FIG. 17B, group D), and 100  $\mu$ M  $N^G$ -monomethyl-L-arginine acetate produced a complete block ( $0.0001 \pm 0.0002$  pmol/mg/min, N=2). These enzymatic data demonstrate that DNOS is a  $Ca^{2+}$ /calmodulin-dependent nitric oxide synthase.

Northern blot analysis indicated a 5.0 kb dNOS transcript which was expressed predominantly in adult fly heads but not bodies (FIG. 18A). More sensitive RT-PCR experiments as described in Example 13, however, detected dNOS message in poly(A)<sup>+</sup> RNA from fly bodies. Neuronal NOS genes from mice and humans produce two alternatively spliced transcripts, the shorter one of which yields a protein containing a 105 amino acid in-frame deletion (residues 504-608 in mouse or rat neuronal NOS) (Ogura, T., *Biochem. Biophys. Res. Commun.*, 193: 1014-1022 (1993)). RT-PCR amplification of *Drosophila* head mRNA produced two DNA fragments: the 444 bp fragment corresponded to vertebrate long form and the 129 bp fragment corresponded to vertebrate short form (FIG. 18B). Conceptual translation of the 129 bp sequence confirmed a splicing pattern identical to that for the nNOS gene (FIG. 18C). Presence of the short NOS isoform in *Drosophila* strengthens the notion that it may play an important role in NOS biochemistry.

The discovery of a NOS homolog in *Drosophila* provides definitive proof that invertebrates produce NO and, as suggested by recent reports, most likely use it for intercellular signaling. These data also suggest that a NOS gene was present in an ancestor common to vertebrates and arthropods, implying that NOS has existed for at least 600 million years. Thus, it is expected that NOS genes are prevalent throughout the animal kingdom.

Consistent with this view are existing histochemical data. NOS activity has been detected in several invertebrate tissue extracts: in *Lymulus polyphemus* Radomski, M. W., *Philos. Trans. R. Soc. Lond. B. Biol. Sci.*, 334: 129-133 (1992)), in the locust brain (Elphick, M. R., et al., *Brain Res.*, 619: 344-346 (1993)), in the salivary gland of *Rhodnius prolixus* (Ribeiro, J. M. C., et al., *FEBS Lett.*, 330: 165-168 (1993)(34)) and in various tissues of *Lymnaea stagnalis* (Elofsson, R., et al., *Neuro Report*, 4: 279-282 (1993)). Applications of NOS inhibitors or NO-generating substances have been shown to modulate the activity of buccal motoneurons in *Lymnaea stagnalis* (Elofsson, R., et al., *Neuro Report*, 4: 279-282 (1993)) and the oscillatory dynamics of olfactory neurons in

procerebral lobe of *Limax maximus* (Gelperin, A., *Nature*, 369: 61-63 (1994)). NADPH-diaphorase staining, a relatively specific indicator of NOS protein in fixed vertebrate tissue samples (Dawson, T. M., et al., *Proc. Natl. Acad. Sci. USA*, 88: 7797 (1991); Hope, B. T., et al., *Proc. Natl. Acad. Sci. USA*, 88: 2811 (1991)), also has suggested the presence of NOS in *Drosophila* heads (Muller, U., *Naturwissenschaften*, 80: 524-526 (1993)). The present molecular cloning of dNOS considerably strengthens the validity of these observations.

Sophisticated genetic analyses of NOS function are available in *Drosophila*. Classical genetics will allow the creation of point mutations and deletions in dNOS, resulting in full or partial loss of dNOS function. Such mutations will permit detailed studies of the role of NOS during development.

The invention further relates to isolated DNA that are characterized by their ability to encode a polypeptide of the amino acid sequence in FIG. 16A-16C (SEQ ID NO.: 9) or functional equivalents thereof (i.e., a polypeptide which synthesizes nitric oxide). Isolated DNA meeting this criteria comprise amino acids having sequences homologous to sequences of mammalian NOS gene products (i.e., neuronal, endothelial and macrophage NOSs). The DNA sequence represented in SEQ ID NO.: 25 is an example of such an isolated DNA. Isolated DNA meeting these criteria also comprise amino acids having sequences identical to sequences of naturally occurring dNOS or portions thereof, or variants of the naturally occurring sequences. Such variants include mutants differing by the addition, deletion or substitution of one or more residues, modified nucleic acids in which one or more residues is modified (e.g., DNA or RNA analogs), and mutants comprising one or more modified residues.

Such nucleic acids can be detected and isolated under high stringency conditions or moderate stringency conditions, for example. "High stringency conditions" and "moderate stringency conditions" for nucleic acid hybridizations are explained on pages 2.10.1-2.10.16 (see particularly 2.10.8-11) and pages 6.3.1-6 in *Current Protocols in Molecular Biology* (Ausubel, F. M. et al., eds, Vol. 1, Suppl. 26, 1991), the teachings of which are incorporated herein by reference. Factors such as probe length, base composition, percent mismatch between the hybridizing sequences, temperature and ionic strength influence the stability of nucleic acid hybrids. Thus, high or moderate stringency conditions can be determined empirically, depending in part upon the characteristics of the known DNA to which other unknown nucleic acids are being compared for homology.

Isolated DNA that are characterized by their ability to encode a polypeptide of the amino acid sequence in FIG. 16A-16C, encode a protein or polypeptide having at least one function of a *Drosophila* NOS, such as a catalytic activity (e.g., synthesis of nitric oxide) and/or binding function (e.g., putative heme, calmodulin, FMN, FAD and NADPH binding). The catalytic or binding function of a protein or polypeptide encoded by hybridizing nucleic acid may be detected by standard enzymatic assays for activity or binding (e.g., assays which monitor conversion of L-arginine to L-citrulline). Functions characteristic of dNOS may also be assessed by in vivo complementation activity or other suitable methods. Enzymatic assays, complementation tests, or other suitable methods can also be used in procedures for the identification and/or isolation of nucleic acids which encode a polypeptide having the amino acid sequence in FIG. 16A-16C or functional equivalents thereof.

The present invention will now be illustrated by the following examples, which are not intended to be limiting in any way.

The following materials and methods were used in the work described in Examples 1 and 2.

#### Expression Cloning of dCREB1 and dCREB2

Standard protocols for expression cloning by DNA-binding (Ausubel, F., *Current Protocols in Molecular Biology*, John Wiley and Sons, New York, 1994; Singh, H. et al., *Cell*, 52: 415-423 (1988)) were followed except as noted. A double-stranded, 3xCRE oligonucleotide was synthesized and cloned between the XbaI and KpnI sites of pGEM7Zf+ (Promega). The sequence of one strand of the oligonucleotide was 5'CGTCTAGATCTATGACTGAATATGACGTAATATGACGTAATGGTACCA GATCTGGCC 3' (SEQ ID NO.: 17), with the CRE sites underlined. The oligonucleotide was excised as a BglII/HindIII fragment and labeled by filling-in the overhanging ends with Klenow fragment in the presence of [ $\alpha$ - $^{32}$ P]dGTP, [ $\alpha$ - $^{32}$ P]dCTP and unlabeled dATP and dTTP (Ausubel, F., *Current Protocols in Molecular Biology*, John Wiley and Sons, New York, 1994). Just prior to use, the labeled fragment was pre-absorbed to blank nitrocellulose filters to reduce background binding. All other steps were as described (Ausubel, F., *Current Protocols in Molecular Biology*, John Wiley and Sons, New York, 1994). After secondary and tertiary lifts, positive clones were subcloned into pKS+ (Stratagene) and sequenced.

#### Gel Shift Analysis

Gel-mobility shift assays were performed as in Ausubel, F., *Current Protocols in Molecular Biology*, John Wiley and Sons, New York, 1994, with the following modifications. The 4% polyacrylamide gel (crosslinking ratio 80:1) was cast and run using 5x Tris-glycine buffer (Ausubel, F., *Current Protocols in Molecular Biology*, John Wiley and Sons, New York, 1994) supplemented with 3 mM MgCl<sub>2</sub>. The oligonucleotides used as the DNA probes were boiled and slowly cooled to room temperature at a concentration of 50  $\mu$ g/ml in 0.1 M NaCl. 50 ng of double-stranded probe was end-labeled using polynucleotide kinase in the presence of 100  $\mu$ Ci of [ $\gamma$ - $^{32}$ P] ATP. The double-stranded oligonucleotides were purified on a native polyacrylamide gel and used in a mobility shift assay at about 0.5 ng/reaction.

For dCREB2, the original dCREB2-b cDNA was subcloned and subjected to site-directed mutagenesis to introduce restriction sites immediately 5' and 3' of the open reading frame. This open reading frame was subcloned into the pET11A expression vector (Novagen) and used to induce expression of the protein in bacteria. The cells containing this vector were grown at 30° C. to an approximate density of 2x10<sup>8</sup>/ml and heat-induced at 37° C. for 2 hours. The cells were collected by centrifugation and lysed according to Buratowski, S. et al., *Proc. Natl. Acad. Sci. USA*, 88: 7509-7513 (1991). The crude extract was clarified by centrifugation and loaded onto a DEAE column previously equilibrated with 50 mM Tris HCl, pH 8.0, 10% sucrose, 100 mM KCl. Step elutions with increasing amounts of KCl in the same buffer were used to elute the dCREB2-b protein, which was assayed using the gel mobility-shift assay. The peak fraction was dialyzed against the loading buffer and used in the binding experiment. The specific competitor that was used was the wild-type CRE oligonucleotide. The sequence of one strand of the double-stranded oligonucleotides used in the gel shift analysis are listed. For the first two oligonucleotides, wild-type and mutant CREs are underlined.

Wild-type 3xCRE (SEQ ID NO.: 18):  
5' AAATGACGTAACGGAAATGACGTAACGGAAATGACGTAACG 3';

5 Mutant 3xmCRE (SEQ ID NO.: 19):  
5' AAATGAATTAACGGAAATGAATTAACGGAAATGAATTAACG 3';

Nonspecific competitor #1 (SEQ ID NO.: 20):  
5' TGCACGGGTTTTTCGACGTTCACTGGTAGTGTCTGATGAGGCCGAAAG  
GCCGAAACGCGATGCCATAACCACCACGCTCAG 3';

10 Nonspecific competitor #2 (SEQ ID NO.: 21):  
5' TCGACCCACAGTTTTTCGGGTTTTTCGAGCAAGTCTGCTAGTGTCTGATG  
AGGCCGAAAGGCCGAAACGCGAAGCCGTATTGCACCACGCTCATCGAGAA  
GGC 3';

15 Nonspecific competitor #3 (SEQ ID NO.: 22):  
5' CTAGAGCTTGCAAGCATGCTTGCAAGCAAGCATGCTTGCAAGCATGC  
TTGCAAGC 3';

Nonspecific competitor #4 (SEQ ID NO.: 23):  
5' CTCTAGAGCGTACGCAAGCGTACGCAAGCGTACG 3'

20 For dCREB1, heat-induced bacterial extracts (Ausubel, F., *Current Protocols in Molecular Biology*, John Wiley and Sons, New York, 1994) were made from the original phage clone integrated by lysogeny. Extract from a bacteria lysogenized with another plaque (which did not bind to CRE sites) from the screen was used as a negative control. Competition experiments were done using a 4-100 fold molar excess (relative to the probe) of unlabeled, wild-type CRE oligonucleotides or unlabeled, mutant CRE oligonucleotides.

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#### Northern Blots

Total head and body RNA was isolated from flies according to the protocol of Drain, P. et al., *Neuron*, 6:71-82 (1991). Total RNA from all other developmental stages was a gift from Eric Schaeffer. All RNA samples were selected twice on oligo-dT columns (5 Prime-3 Prime) to isolate poly A+ RNA. Two  $\mu$ g of poly A+ RNA was fractionated on 1.2% formaldehyde-formamide agarose gels, transferred to nitrocellulose and probed using a uniformly labeled, strand-specific, antisense RNA (aRNA) probe. The template for the synthesis of aRNA was one of the partial cDNA clones isolated from the library screen (pJY199). This cDNA contained the carboxyl-terminal 86 amino acids of the dCREB2-b protein and about 585 bp of 3' untranslated mRNA. All Northern blots were washed at high stringency (0.1% SDS, 0.1xSSC, 65° C.).

45

#### In situ Hybridization to Tissue Sections

50 Frozen frontal sections were cut and processed under RNase-free conditions, essentially as described in Nighorn, A. et al., *Neuron*, 6:455-467 (1991), with modifications for riboprobes as noted here. Digoxigenin-labeled riboprobes were made from pJY199 using the Genius kit (Boehringer-Mannheim). One  $\mu$ g of Xba-linearized template and T3 RNA polymerase was used to make the antisense probe, while one  $\mu$ g of EcoRI-linearized template together with T7 RNA polymerase was used for the control sense probe. Alkaline hydrolysis (30 minutes at 60° C.) was used to reduce the average probe size to about 200 bases. The hydrolyzed probe was diluted 1:250 in hybridization solution (Nighorn, A. et al., *Neuron*, 6:455-467 (1991)), boiled, quickly cooled on ice, added to the slides and hybridized at 42° C. overnight. The slides were then treated with RNase A (20  $\mu$ g/ml RNase A in 0.5 M NaCl/10 mM Tris pH8 for 1 hour at 37° C.) prior to two 50° C. washes. Digoxigenin detection was as described.

65

Reverse Transcription Coupled With the Polymerase  
Chain Reaction (RT-PCR) Analysis of dCREB2 and  
Identification of Alternatively Spliced Exons

The template for reverse transcription coupled with the polymerase chain reaction (RT-PCR) was total RNA or poly A+ RNA isolated from *Drosophila* heads as in Drain, P. et al., *Neuron*, 6: 71-82 (1991). Total RNA used was exhaustively digested with RNase-free DNase 1 (50 µg of RNA digested with 50 units of DNase I for 60-90' at 37° C. followed by phenol, phenol/chloroform extraction, and ethanol precipitation) prior to use. Results from separate experiments indicate that this DNase-treatment effectively eliminates the possibility of PCR products derived from any contaminating genomic DNA. Two rounds of selection using commercial oligo-dT columns (5 Prime-3 Prime) were used to isolate poly A+ RNA from total RNA. The template for an individual reaction was either 100-200 ng of total RNA, or 10-20 ng of poly A+ RNA.

The RT-PCR reactions were performed following the specifications of the supplier (Perkin-Elmer) with a "Hot Start" modification (Perkin-Elmer RT-PCR kit instructions). All components of the RT reaction, except the rTth enzyme, were assembled at 75° C., and the reaction was initiated by adding the enzyme and lowering the temperature to 70° C. At the end of 15 minutes, the preheated (to 75° C.) PCR components (including trace amounts of [ $\alpha$ -<sup>32</sup>P]dCTP) were added quickly, the reaction tubes were put into a pre-heated thermocycler, and the PCR amplification begun. Cycling parameters for reactions (100 µl total volume) in a Perkin-Elmer 480 thermocycler were 94° C. for 60 seconds, followed by 70° C. for 90 seconds. For reactions (50 µl) in an MJ Minicycler the parameters were 94° C. for 45 seconds and 70° C. for 90 seconds.

All primers used in these procedures were designed to have 26 nucleotides complementary to their target sequence. Some primers had additional nucleotides for restriction sites at their 5' ends to facilitate subsequent cloning of the products. Primers were designed to have about 50% GC content, with a G or C nucleotide at their 3' most end and with no G/C runs longer than 3. For RT-PCR reactions with a given pair of primers, the Mg<sup>+2</sup> concentration was optimized by running a series of pilot reactions, at Mg<sup>+2</sup> concentrations ranging from 0.6 mM to 3.0 mM. Reaction products were analyzed on denaturing urea-polyacrylamide gels by autoradiography. Any product that appeared larger than the band predicted from the cDNA sequence was purified from a preparative native gel, re-amplified using the same primers, gel-purified, subcloned and sequenced.

To verify that a given RT-PCR product was truly derived from RNA, control reactions were run to show that the appearance of the product was eliminated by RNase A treatment of the template RNA. Also, products generated from reactions using total RNA as the template were re-isolated from reactions using twice-selected polyA+ RNA as template.

#### Plasmids

Expression constructs for transient transfection experiments in *Drosophila* were made in the expression vector pAct5CPPA (Han, K. et al., *Cell*, 56: 573-583 (1989)) or in pAcQ. pAcQ is a close derivative of pAct5CPPA in which the XbaI site at the 5' end of the 2.5 kb actin promoter fragment was destroyed and additional sites were inserted in the

polylinker. pAc-dCREB1 was made by subcloning a KpnI-SacI fragment containing the complete dCREB1 open reading frame (from a cDNA subcloned into pKS+) into pAct5CPPA. pAc-PKA was constructed by subcloning an EcoRV fragment encoding the *Drosophila* PKA catalytic subunit (Foster, J. L. et al., *J. Biol. Chem.*, 263: 1676-1681 (1988)) from a modified pHSREM1 construct (Drain, P. et al., *Neuron*, 6: 71-82 (1991)) into pAct5CPPA. To make the 3xCRE-lacZ reporter construct for *Drosophila* cell culture, the double-stranded, wild-type 3xCRE oligonucleotide used in the gel shift analysis was cloned into the KpnI-XbaI backbone of HZ50PL (Hiromi, Y. and W. J. Gehring, *Cell*, 50: 963-974 (1987)), a reporter construct made for enhancer testing which has cloning sites in front of a minimal hsp70 promoter-lacZ fusion gene.

RSV-dCREB2-a was constructed in a long series of cloning steps. Essentially, the activator-encoding open reading frame was first reconstructed on the plasmid pKS+ by sequentially adding each of the three exons (exons 2, 4 and 6) into the original cDNA of dCREB2-b, which had been subcloned from phage DNA into pKS+. Site-directed mutagenesis was used to introduce unique restriction enzyme sites both 5' and 3' of the dCREB2-b open reading frame, and these sites facilitated the subcloning process and allowed removal of 5' and 3' untranslated sequences. Once the activator was assembled, the resulting open reading frame was sequenced to confirm the cloning steps and moved into a modified RSV vector which contained a polylinker located between the RSV promoter and the SV40 polyadenylation sequences (RSV-0). RSV-dCREB2-b was made by moving the original dCREB2-b cDNA (which had been subcloned into pKS+) into RSV-0.

Other constructs used in experiments were: pCaE (pMtC) (Mellon, P. L. et al., *Proc. Natl. Acad. Sci. USA*, 86: 4887-4891 (1989)), which contains the cDNA for mouse PKA catalytic subunit cloned under the mouse metallothionein 1 promoter; RSV-βgal (Edlund, T. et al., *Science*, 230: 912-916 (1985)), which expresses the lacZ gene under control of the Rous sarcoma long terminal repeat promoter (Gorman, C. M. et al., *Proc. Natl. Acad. Sci. USA*, 79: 6777-6781 (1982)). RSV-CREB (Gonzalez, G. A. et al., *Nature*, 337: 749-752 (1989)) is a CREB cDNA fragment containing the 341-amino acid open reading frame under the RSV LTR-promoter in RSV-SG, and the D(-71) CAT reporter (Montminy, M. R. et al., *Proc. Natl. Acad. Sci. USA*, 83: 6682-6686 (1986)) which is a fusion of a CRE-containing fragment of the rat somatostatin promoter and the bacterial CAT coding region.

#### F9 Cell Culture and Transfection

Undifferentiated F9 cells were maintained and transfected using the calcium phosphate method as described in Darrow, A. L. et al., "Maintenance and Use of F9 Teratocarcinoma Cells", In *Meth. Enzymol.*, v. 190 (1990), except that chloroquine was added to 100 mM immediately before transfection and precipitates were washed off ten hours after transfection, at which time the dishes received fresh chloroquine-free medium. Amounts of DNA in transfections were made equivalent by adding RSV-0 where required. Cells were harvested 30 hours after transfection. Extracts were made by three cycles of freeze/thawing, with brief vortexing between cycles. Particulate matter was cleared from extracts by ten minutes of centrifugation in the cold. β-galactosidase assays were performed as described in Miller, J. H., *Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1972. CAT assays were performed as described in Sheen, J. Y. and B. Seed, *Gene*, 67: 271-277

(1988) using aliquots of extract heat-treated at 65° C. for ten minutes and centrifuged for ten minutes to remove debris. Results reported are from three experiments run on different days with at least three dishes per condition within each experiment. Error bars represent standard error of the mean, with error propagation taken into account (Grossman, M. and H. W. Norton, *J. Hered.*, 71: 295-297 (1980)).

#### *Drosophila* Cell Culture and Transient Transfection

Schneider L2 cells in Schneider's medium (Sigma) supplemented with 10% fetal bovine serum (FBS) or Kc167 cells in D-22 medium (Sigma) supplemented with 10% FBS, were transfected by the calcium phosphate method essentially as described in Krasnow, M. A. et al., *Cell*, 57: 1031-1043 (1989), with the following differences. Kc167 cells were plated at  $2 \times 10^6$  cells/ml and chloroquine was added to a final concentration of 100 mM immediately prior to transfection. A total of 10  $\mu$ g of plasmid DNA per dish was used for L2 transfections and 25  $\mu$ g per dish for Kc167 transfections. DNA masses in transfections were made equivalent with pGEM7Zf+ where required. Precipitates were left undisturbed on L2 cells until harvest, but for Kc167 cells the original medium was replaced with fresh, chloroquine-free medium after twelve hours. Cells were harvested thirty-six to forty-eight hours after transfection. Extracts were made and enzymatic assays performed as described above for F9 cells. Results reported for transfections are averages of at least three experiments run on different days, with at least duplicate dishes for each condition within experiments. Error bars represent standard error of the mean, with error propagation taken into account (Grossman, M. and H. W. Norton, *J. Hered.*, 71: 295-297 (1980)).

#### $\beta$ -galactosidase ( $\beta$ gal) and Chloramphenicol Acetyl Transferase (CAT) Assays

$\beta$ -galactosidase assays were run and activity calculated as described in Miller, J. H., *Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1972. CAT assays were performed essentially according to Sheen, J. Y. and B. Seed, *Gene*, 67: 271-277 (1988) using supernatants from heat-treated aliquots of extracts (65° C. for 10 minutes and then centrifuged for 10 minutes). Relative activity was calculated according to Sheen, J. Y. and B. Seed, *Gene*, 67: 271-277 (1988).

#### PKA-Responsive Transcriptional Activation by dCREB2-a

F9 cells were transiently transfected with 10  $\mu$ g of D(-71) CAT plasmid as a CRE-directed reporter. 5  $\mu$ g of RSV- $\beta$ gal reporter was included in each dish as a normalization control for transfection efficiency. Different groups received 8  $\mu$ g of dCREB2-a expression vector and 4  $\mu$ g of PKA expression vector, separately or in combination. Results are expressed as CAT/Pgal enzyme activity ratios, standardized to values obtained with PKA-transfected dishes.

#### Transcriptional Effect of dCREB2-b and a Mutant Variant on PKA-Responsive Activation by dCREB2-a

F9 cells were transiently cotransfected with 10  $\mu$ g of D(-71) CAT along with the indicated combinations of the following expression constructs: RSV-dCREB2-a (5  $\mu$ g); pMtC (2  $\mu$ g); RSV-dCREB2-b (5  $\mu$ g); and RSV-mLZ-

dCREB2-b, which expresses a leucine-zipper mutant of dCREB2-b (5  $\mu$ g). The DNA mass for each dish was made up to 27  $\mu$ g with RSV-0. Other experimental conditions are as described above under "PKA-Responsive Transcriptional Activation by dCREB2-a".

#### Transcriptional Activation of a CRE Reporter Gene by dCREB1 in *Drosophila* Schneider L2 Cell Culture

The cells were transiently transfected with a dCREB1 expression construct (1  $\mu$ g), with or without a construct which expresses *Drosophila* PKA. 3 $\times$ CRE- $\beta$ gal reporter (1  $\mu$ g) and the normalization Ac-CAT reporter (1  $\mu$ g) were included in each dish. Expression vectors not present in particular dishes were replaced by pACQ.

#### Example 1

##### Isolation and Characterization of dCREB2

Two different genes were isolated in a DNA-binding expression screen of a *Drosophila* head cDNA library using a probe containing three CRE sites (3 $\times$ CRE). Many clones were obtained for the dCREB2 gene, while only one clone was obtained for dCREB1. The dCREB2 clones had two alternatively-spliced open reading frames, dCREB2-b and dCREB2-c (see FIG. 2). These differed only in the presence or absence of exon 4 and in their 5' and 3' untranslated regions. The inferred translation product of dCREB2-b showed very high sequence similarity to the amino acid sequences of the basic region/leucine zipper (bZIP) domains of mammalian CREB (SEQ ID NO.: 4), CREM (SEQ ID NO.: 5) and ATF-1 (SEQ ID NO.: 6) (see FIG. 1B).

Chromosomal in situ hybridization using a dCREB2 probe localized the gene to a diffuse band at 17A2 on the X chromosome, a region which contains several lethal complementation groups (Eberl, D. F. et al., *Genetics*, 30: 569-583 (1992)).

To determine the DNA binding properties of dCREB2-b, the DNA binding activity of dCREB2-b was assayed using a gel mobility-shift assay. Bacterial extracts expressing the dCREB2-b protein retarded the migration of a triplicated CRE probe (3 $\times$ CRE). The protein had lower, but detectable, affinity for a mutated 3 $\times$ CRE oligonucleotide. Competition experiments using unlabeled competitor oligonucleotides showed that the binding of dCREB2-b to 3 $\times$ CRE was specific with higher affinity for CRE sites than to nonspecific DNA. Together with the conserved amino acid sequence, this functional similarity suggested that dCREB2 was a CREB family member.

The expression pattern of dCREB2 was determined by Northern blot analysis of poly A+RNA from various developmental stages. A complex pattern with at least 12 different transcript sizes was found. Two bands of approximately 0.8 and 3.5 kb were common to all of the stages. The adult head contained transcripts of at least six sizes (0.8, 1.2, 1.6, 1.9, 2.3 and 3.5 kb). In situ hybridization to RNA in *Drosophila* head tissue sections showed staining in all cells. In the brain, cell bodies but not neuropil were stained.

dCREB2 has alternatively-spliced forms. Initial transfection experiments showed that the dCREB2-c isoform was not a PKA-responsive transcriptional activator. This information, together with the complex developmental expression pattern and the known use of alternative splicing of the CREM gene to generate PKA-responsive activators (Foulkes, N. and P. Sassone-Corsi, *Cell*, 68: 411-414 (1992); Foulkes, N. S. et al.,



*Nature*, 355: 80-84 (1992)) suggested that additional domains might be required to code for an activator.

Reverse transcription coupled with the polymerase chain reaction (RT-PCR) was used to identify new exons. Comparison of the genomic DNA sequence with that of cDNAs indicated the general exon/intron organization and assisted in the search for additional exons. Sense and antisense primers spaced across the dCREB2-b cDNA were synthesized and used pairwise in RT-PCR reactions primed with *Drosophila* head RNA. Reactions with primers in exons 5 and 7 (see FIG. 2) generated two products, one with the predicted size (compared with the cDNA clones) and one that was larger. The larger fragment was cloned and its sequence suggested the presence of exon 6 (see FIG. 1A; SEQ ID NO.: 1). A primer within exon 6 was synthesized, end-labeled and used to screen a *Drosophila* head cDNA library. Two clones were isolated, sequenced and found to be identical. This splicing isoform, dCREB2-d, confirmed the splice junctions and exon sequence inferred from the RT-PCR products.

Initial attempts to isolate exon 2 proved difficult. The genomic sequence that separated exons 1 and 3 (see FIG. 2) was examined and one relatively extensive open reading frame (ORF) was identified. Three antisense primers, only one of which lay inside this ORF, were synthesized based on the intron sequence. Three sets of RT-PCR reactions were run in parallel, each using one of the three antisense primers and a sense primer in exon 1. Only the reaction that used the antisense primer in the ORF produced a PCR product. The sequence of this product matched a continuous stretch of nucleotides from the genomic sequence, extending 3' from exon 1 past the splice junction in the dCREB2-b cDNA to the location of the antisense primer in the ORF. This fragment suggested that exon 1 might be extended in some mRNAs by use of an alternative 5' splice site located 3' to the site used to make dCREB2-b. Based on the newly-identified exon sequences, a sense primer was made. This primer was used with an antisense primer in exon 3 to generate a new product whose sequence established the location of the new 5' splice site. The sequence added to exon 1 by alternative 5' splice site selection is denoted exon 2. The exon 2 sequence also showed that the same 3' splice site was used both for the original cDNA and for exon 2. To independently verify this alternative splicing pattern, RT-PCR was carried out with a primer that spanned the 3' splice junction of exon 2 and a primer in exon 1. The sequence of the product corroborated the splice junctions of exon 2 shown in FIG. 1A (SEQ ID NO.: 1).

To determine if exons 2 and 6 could be spliced into the same molecule, an RT-PCR reaction was carried out with primers in exons 2 and 6. The reaction produced a product of the size predicted by coordinate splicing of these two exons and the identity of this product was confirmed by extensive restriction analysis.

dCREB2 is a *Drosophila* CREB/ATF gene. FIG. 1A shows the DNA sequence (SEQ ID NO.: 1) and inferred amino acid sequence (SEQ ID NO.: 2) of dCREB2-a, the longest ORF that can result from the identified alternative splicing events. The indicated translation start site for this ORF is probably authentic because: i) stop codons occur upstream from this ATG in all reading frames in our dCREB2 cDNAs (sequences not shown) ii) this ATG was selected by computer (Sheen, J. Y. and B. Seed, *Gene*, 67: 271-277 (1988)) as the best ribosome binding site in the DNA sequence that contains the ORF; and iii) use of the next ATG in the open reading frame 480 nucleotides downstream would not produce an inferred product that is a PKA-dependent activator (see below). This information does not exclude the possibility that internal translation initiation sites may be used in this transcript, as

happens in the CREM gene's S-CREM isoform (Delmas, V. et al., *Proc. Natl. Acad. Sci. USA*, 89: 4226-4230 (1992)).

The dCREB2-a open reading frame predicts a protein of 361 amino acids with a carboxyl-terminal bZIP domain (SEQ ID NO.: 3) highly homologous to those of mammalian CREB (SEQ ID NO.: 4) and CREM (SEQ ID NO.: 5) (see FIG. 1B). The inferred dCREB2-a product has a small region of amino acids containing consensus phosphorylation sites for PKA, calcium/calmodulin-dependent kinase II (CaM kinase II) and protein kinase C (PKC) at a position similar to that of the P-box in CREB, CREM and ATF-1. The amino-terminal third of the predicted dCREB2-a is rich in glutamines (including runs of four and five residues). Glutamine-rich activation domains occur in CREB, CREM, and other eukaryotic transcription factors, including some from *Drosophila* (Courey, A. J. and R. Tijan, "Mechanisms of Transcriptional Control as Revealed by Studies of the Human Transcription Factor Sp 1", *In Transcriptional Regulation*, vol. 2, McKnight, S. L. and K. R. Yamamoto (eds.), Cold Spring Harbor Press, Cold Spring Harbor, N.Y., 1992; Mitchell, P. J. and R. Tijan, *Science*, 245: 371-378 (1989)).

A computerized amino acid sequence homology search with the predicted dCREB2-a protein sequence (SEQ ID NO.: 2) identifies CREB, CREM and ATF-1 gene products as the closest matches to dCREB2-a. The homology is particularly striking in the carboxyl-terminal bZIP domain where 49 of 54 amino acids are identical with their counterparts in mammalian CREB (FIG. 1B). The homology is less striking, albeit substantial, in the activation domain. Lower conservation in this domain is also characteristic of the mammalian CREB and CREM genes (Masquillier, D. et al., *Cell Growth Differ.*, 4: 931-937 (1993)).

FIG. 2 shows the exon organization of all of the dCREB2 alternative splice forms that we have detected, both as cDNAs and by RT-PCR. Splice products of dCREB2 fall into two broad categories. One class of transcripts (dCREB2-a, -b, -c, -d) employs alternative splicing of exons 2, 4 and 6 to produce isoforms whose protein products all have the bZIP domains attached to different versions of the activation domain. The second class of transcripts (dCREB2-q, -r, -s) all have splice sites which result in in-frame stop codons at various positions upstream of the bZIP domain. These all predict truncated activation domains without dimerization or DNA binding activity.

Two different dCREB2 isoforms, dCREB2-a and dCREB2-b, have opposite roles in PKA-responsive transcription. The capacity of isoforms of the dCREB2 gene to mediate PKA-responsive transcription was tested in F9 cells. These cells have been used extensively to study CREB-dependent activation because their endogenous cAMP-responsive transcription system is inactive (Gonzalez, G. A. et al., *Nature*, 337: 749-752 (1989); Masson, N. et al., *Mol. Cell Biol.*, 12: 1096-1106 (1992); Masson, N. et al., *Nucleic Acids Res.*, 21: 1163-1169 (1993)). Candidate cAMP-responsive transcription factors, synthesized from expression vectors, were transiently transfected with and without a construct expressing the PKA catalytic subunit. CREB-dependent changes in gene expression were measured using a cotransfected construct that has a CRE-containing promoter fused to a reporter gene.

The product of the dCREB2-a isoform is a PKA-dependent activator of transcription (FIG. 3). Transfection of PKA or dCREB2-a alone gave only modest activation above baseline values. Cotransfection of dCREB2-a and PKA together, however, gave levels of activation 5.4-fold greater than the activation seen with PKA alone.

dCREB2-b did not act as a PKA-dependent transactivator. When transfected together with the reporter and PKA, it

failed to stimulate reporter activity. Instead, it functioned as a direct antagonist of PKA-dependent activation by dCREB2-a (FIG. 4). Cotransfection of equimolar amounts of the dCREB2-a and dCREB2-b expression constructs, along with PKA and the reporter, resulted in a nearly complete block of PKA-dependent activation from the CRE-containing reporter.

The strong homology between the leucine zippers of dCREB2 (SEQ ID NO.: 3), CREB (SEQ ID NO.: 4) and CREM (SEQ ID NO.: 5) (see FIG. 1B) suggested that mutations which abolish CREB dimerization (Dwarki, V. J. et al., *EMBO J*, 9: 225-232 (1990)) should also affect dCREB2 dimerization. The mutant *Drosophila* molecule mLZ-dCREB2-b was made by introducing two single-base changes that convert the middle two leucines of the leucine zipper to valines. An identical mutation in CREB abolishes homodimerization in vitro (Dwarki, V. J. et al., *EMBO J*, 9: 225-232 (1990)). Cotransfected mLZ-dCREB2-b failed to block PKA-dependent activation by dCREB2-a (FIG. 4).

### Example 2

#### Isolation and Characterization of dCREB1

A single cDNA representing the dCREB1 gene was isolated in the same screen of a *Drosophila* lambda gt11 expression library that yielded the dCREB2 cDNAs. The sequence of the dCREB1 cDNA contained a complete open reading frame specifying a 266 amino acid protein with a carboxyl-terminal leucine zipper four repeats long and an adjacent basic region (FIG. 5; SEQ ID NO.: 7). The amino-terminal half of the inferred protein contains an acid-rich activation domain, with glutamate, aspartate and proline residues spaced throughout. dCREB1 has consensus phosphorylation sites for CaM kinase II and PKC throughout its length, but has no predicted phosphoacceptor site for PKA.

Gel shift analysis showed higher-affinity binding of the dCREB1 protein to 3xCRE than to 3xmCRE. Transcriptional activation by dCREB1 was assayed with transient cotransfection experiments using the *Drosophila* L2 and Kc 167 cell culture lines. In L2 cells, dCREB1 activates transcription from CREs, but this effect is not enhanced by cotransfection of PKA (FIG. 6). In Kc167 cells, dCREB1 fails to activate reporter expression either alone or with cotransfected PKA expression constructs.

Genomic Southern blot analysis indicates that dCREB1 is a single copy gene, and chromosomal in situ hybridization shows that it is located at 54A on the right arm of chromosome 2.

These results show that dCREB1 is a non-PKA responsive CREB family member from *Drosophila*.

The following materials and methods were used in the work described in Examples 3 and 4.

#### Isolating Transgenic Flies

EcoRI restriction sites were added (using PCR) just 5' to the putative translation initiation site and just 3' to the translation termination site in the dCREB2-b cDNA. This fragment was sequenced and subcloned into CaSpeR hs43, a mini-white transformation vector which contains the hsp70 promoter, in the orientation so that the dCREB2-b open reading frame is regulated by the hsp70 promoter. Germ-line transformation was accomplished using standard techniques (Spradling, A. C. and G. M. Rubin, *Science*, 218: 341-347 (1982); Rubin, G. M. and A. Spradling, *Science*, 218: 348-353 (1982)). Two transgenic lines, 17-2 and M11-1, each with

one independent P-element insertion were generated and characterized. They appeared normal in general appearance, fertility and viability. These transgenic lines were outcrossed for at least five generations to w(CS10) (Dura, J-M., et al., *J. Neurogenet.*, 9: 1-14 (1993)), which itself had been outcrossed for ten generations to a wild-type (Can-S) stock. This extensive series of outcrossing is necessary to equilibrate the genetic background to that of Canton-S. Flies homozygous for the 17-2 transgene were bred and used for all experiments.

The mutant blocker has been described previously (see Example 1). The mutations were substituted into an otherwise wild-type blocker construct and germ-line transformants were made by injecting into w(isoCJ1) embryos. Flies homozygous for the A2-2 transgene insertion were bred and used for all experiments. w(isoCJ1) is a subline of w(CS10) (see above) carrying isogenic X, 2<sup>nd</sup> and 3<sup>rd</sup> chromosomes and was constructed by Dr. C. Jones in our laboratory. Originally 40 such sublines were w(CS10) using standard chromosome balancer stocks. Olfactory acuity, shock reactivity, learning and three-hr memory after one-cycle training then were assayed in each isogenic subline. As expected, a range of scores among the sublines was obtained. w(isoCJ1) yielded scores that were most like those of w(CS10) on each of these assays. By injecting DNA into the relatively homogeneous genetic background of w(isoCJ1), outcrossing of the resulting germ-line transformants to equilibrate heterogeneous genetic backgrounds was not necessary.

#### Cycloheximide Feeding and Heat-Shock Regimen

For experiments on memory retention after one-cycle training and on retrograde amnesia, flies were fed 35 mM cycloheximide (+CMX; Sigma) in 4% sucrose (w/v) or 4% sucrose alone (-CXM) at 25° C. Groups of 100 flies were placed in feeding tubes (Falcon 2017) containing two 1.0x2.5 cm Whatmann 3MM filter paper strips that were soaked with a total of 250 µl of solution.

For experiments on one day retention after massed or spaced training, flies were fed 35 mM CXM and (w/v) 5% glucose dissolved in 3% ethanol. Groups of 100 flies were placed in feeding tubes (Falcon 2017) containing one 1.0x2.5 cm Whatmann 3MM filter paper strips that was soaked with a total of 126 µl of solution.

For experiments on learning after one-cycle training, olfactory acuity, and shock reactivity, flies were fed a 5% glucose, 3% ethanol solution alone or 35 mM CXM in the glucose/ethanol solution.

The feeding period was limited to 12-14 hrs prior to training, or to the 24-hr retention interval after training. Flies which were fed prior to training were transferred directly to the training apparatus after feeding, subjected to massed or spaced training, then transferred to test tubes containing filter paper strips soaked with 5% glucose during the 24-hr interval. Flies which were fed after training were trained, then transferred immediately to test tubes containing filter paper strips soaked with 5% glucose solution which was laced with 35 mM CXM. Flies remained in the test tubes for the duration of the 24-hr retention interval.

For heat-shock induction, flies were collected within two days of eclosion, placed in glass bottles in groups of about 600, and incubated overnight at 25° C. and 70% relative humidity. The next day, three hours before training, groups of approximately 100 flies were transferred to foam-stoppered glass shell vials containing a strip of filter paper to absorb excess moisture. The vials then were submerged in a 37° C. water bath until the bottom of the foam stopper (inside the vial) was below the surface of the water, thereby insuring that

the flies could not escape heat-shock. The vial remained submerged for 30 min, after which the flies were transferred to a standard food vial for a 3-hr recovery period at 25° C. and 70% relative humidity. Training began immediately after the recovery period.

#### Pavlovian Learning and Memory and Testing

Flies were trained with an automated version of the learning procedure of Tully, T. and W. G. Quinn, *J. Comp. Physiol.*, 157: 263-277 (1985). In brief, flies were trapped in a training chamber, the inside of which was covered with an electrifiable copper grid. Groups of about 100 flies were exposed sequentially to two odors [either octanol (OCT) or methylcyclohexanol (MCH)], which were carried through the training chamber in a current of air, for 60 seconds with 45 seconds rest intervals after each odor presentation. During exposure to the first odor, flies also were subjected to twelve 1.5-second pulses of 60 V DC with a 5-second interpulse interval. After training, flies were transferred to food vials for a particular retention interval. Conditioned odor-avoidance responses then were tested by transferring flies to the choice point of a T-maze, where they were exposed simultaneously to OCT and MCH carried in the distal ends of the T-maze arms and out the choice point on converging currents of air. Flies were allowed to distribute themselves in the T-maze arms for two minutes, after which they were trapped in their respective arms, anesthetized and counted. The “percent correct” then was calculated as the number of flies avoiding the shock-paired odor (they were in the opposite T-maze arm) divided by the total number of flies in both arms. (The number of flies left at the choice point, which usually was less than 5%, were not included in this calculation). Finally, a performance index (PI) was calculated by averaging the percent corrects of two reciprocal groups of flies—one where OCT and shock were paired, the other where MCH and shock were paired—and then by normalizing the average so that a PI=0 represented a 50:50 distribution in the T-maze and a PI=100 represented 100% avoidance of the shock-paired odor. For these studies, three different training protocols were used: 1. One-cycle training consisted of the training session just described. 2. Massed training consisted of 10 of these training cycles delivered one right after the other. 3. Spaced training consisted of 10 training cycles with a 15-min rest interval between each. One-cycle training was used to assay learning, while massed and spaced was used to assay consolidated memories.

#### Olfactory Acuity and Shock Reactivity

Odor avoidance responses to OCT or to MCH at two different concentrations—one ( $10^0$ ) usually used in conditioning experiments and a 100-fold ( $10^{-2}$ ) dilution thereof—were quantified in various groups of flies in the absence of heat shock and 3 hr or 24 hr after heat shock with the method of Boynton, S. and T. Tully, *Genetics*, 131: 655-672 (1992). Briefly, flies are placed in a T-maze and given a choice between an odor and air. The odors are naturally aversive, and flies usually choose air and avoid the T-maze arm containing the odor. For shock reactivity, flies are given a choice between an electrified grid in one T-maze arm, and an unconnected grid in the other. After the flies have distributed themselves, they are anesthetized, counted and a PI is calculated.

#### Statistical Analyses of Behavioral Data

Since each PI is an average of two percentages, the Central Limit Theorem predicts that they should be distributed nor-

mally (see Sokal, R. R. and F. J. Rohlf, *Biometry*, 2nd Edition, W.H. Freeman and Company, New York (1981)). This expectation was shown to be true by an empirical determination with data from Tully, T. and W. G. Quinn, *J. Comp. Physiol.*, 157: 263-277 (1985) and Tully, T. and D. Gold, *J. Neurogenet.*, 9: 55-71 (1993). Thus, untransformed (raw) data were analyzed parametrically with JMP2.1 statistical software (SAS Institute Inc., Cary N.C.). Since preliminary experiments preceded all of the experiments summarized herein, all pairwise comparisons were planned. To maintain an experimentwise error rate of  $\alpha=0.05$ , the critical P values for these individual comparisons were adjusted accordingly (Sokal, R. R. and F. J. Rohlf, *Biometry*, 2nd Edition, W.H. Freeman and Company, New York (1981)) and are listed below for each experiment.

All experiments were designed in a balanced fashion with N=2 PIs per group collected per day; then replicated days were added to generate final Ns. In each experiment, the experimenter (M.D.) was blind to genotype.

A. One-day memory in wild-type flies fed CXM before or immediately after massed or spaced training (FIG. 8): PIs from these four drug treatments (-CXM before, -CXM after, +CXM before and +CXM after) and two training procedures (massed and spaced) were subjected to a TWO-WAY ANOVA with DRUG ( $F_{(3,56)}=8.93$ ;  $P<0.001$ ) and TRAINING ( $F_{(1,56)}=18.10$ ,  $P<0.001$ ) as main effects and DRUG×TRAIN ( $F_{(3,56)}=4.68$ ,  $P=0.006$ ) as the interaction term. P values from subsequent planned comparisons are summarized in FIG. 8. The six planned comparisons were judged significant if  $P\leq 0.01$ .

B. One-day memory after massed or spaced training in dCREB2-b transgenic flies (FIGS. 9A and 9B): In experiments with the 17-2 transgenic line, PIs from two strains (Can-S and 17-2) and four training-regimens (spaced -hs, spaced +hs, massed -hs and massed +hs) were subjected to a TWO-WAY ANOVA with STRAIN ( $F_{(1,40)}=1.57$ ;  $P=0.22$ ) and TRAINING-regimen ( $F_{(3,40)}=25.81$ ,  $P<0.001$ ) as main effects and STRAIN×TRAIN ( $F_{(3,40)}=6.62$ ,  $P=0.001$ ) as the interaction term. A similar analysis was done with data from the M 11-1 transgenic line, yielding STRAIN ( $F_{(1,40)}=2.81$ ;  $P=0.10$ ), TRAINING-regimen ( $F_{(3,40)}=11.97$ ,  $P<0.001$ ) and STRAIN×TRAIN ( $F_{(3,40)}=3.37$ ,  $P=0.03$ ) effects. P values from subsequent planned comparisons are summarized in FIGS. 9A and 9B. In each experiment, the seven planned comparisons were judged significant if  $P\leq 0.01$ .

C. Learning after one-cycle training in 17-2 transgenic flies (FIG. 9C): PIs from two strains (Can-S and 17-2) and three heat-shock regimens [-hs, +hs (3 hr) and +hs (24 hr)] were subjected to a TWO-WAY ANOVA with STRAIN ( $F_{(1,30)}=0.69$ ;  $P=0.41$ ) and HEAT-shock regimen ( $F_{(2,30)}=10.29$ ,  $P<0.001$ ) as main effects and STRAIN×HEAT ( $F_{(2,30)}=0.71$ ,  $P=0.50$ ) as the interaction term. P values from subsequent planned comparisons are summarized in FIG. 9C. The three planned comparisons were judged significant if  $P\leq 0.02$ .

D. One-day memory after spaced training in A2-2 transgenic flies (FIG. 10): PIs from these three strains [w(iso)CJ1], 17-2 and A2-2] and two heat-shock regimens [-hs and +hs (3 hr)] were subjected to a TWO-WAY ANOVA with STRAIN ( $F_{(2,30)}=9.43$ ,  $P<0.001$ ) and HEAT-shock regimen ( $F_{(1,30)}=9.84$ ,  $P=0.004$ ) as main effects and STRAIN×HEAT ( $F_{(2,30)}=5.71$ ,  $P=0.008$ ) as the interaction term. P values from subsequent comparisons are summarized in FIG. 10. The six planned comparisons were judged significant if  $P\leq 0.01$ .

E. Olfactory acuity in 17-2 flies (Table): PIs from these two strains (Can-S and 17-2), four different odor-levels (OCT- $10^0$ , OCT- $10^{-2}$ , MCH- $10^0$  and MCH- $10^{-2}$ ) and three heat-shock regimens [-hs, +hs (3 hr) and +hs (24 hr)] were sub-

jected to a THREE-WAY ANOVA with STRAIN ( $F_{(1,184)}=0.12$ ,  $P=0.73$ ), ODOR-level ( $F_{(3,184)}=126.77$ ,  $P<0.001$ ) and HEAT-shock regimen ( $F_{(2,184)}=3.55$ ,  $P=0.03$ ) as main effects, STRAIN×ODOR ( $F_{(3,184)}=1.23$ ,  $P=0.30$ ), STRAIN×HEAT ( $F_{(2,184)}=0.33$ ,  $P=0.72$ ) and ODOR×HEAT ( $F_{(6,184)}=3.14$ ,  $P=0.006$ ) as two-way interaction terms and STRAIN×ODOR×HEAT ( $F_{(6,184)}=0.48$ ,  $P=0.83$ ) as the three-way interaction term. P values from subsequent planned comparisons are summarized in the Table. The twelve planned comparisons were significant if  $P\leq 0.005$ .

F. Shock reactivity in 17-2 flies (Table): PIs from these two strains (Can-S and 17-2), two shock groups (60V and 20V) and three heat-shock regimens [-hs, +hs (3 hr) and +hs (24 hr)] were subjected to a THREE-WAY ANOVA with STRAIN ( $F_{(1,84)}=0.50$ ,  $P=0.48$ ), SHOCK ( $F_{(1,84)}=97.78$ ,  $P<0.001$ ) and HEAT-shock regimen ( $F_{(2,84)}=3.36$ ,  $P=0.04$ ) as main effects, STRAIN×SHOCK ( $F_{(1,84)}=1.12$ ,  $P=0.29$ ), STRAIN×HEAT ( $F_{(2,84)}=1.06$ ,  $P=0.35$ ) and SHOCK×HEAT ( $F_{(2,84)}=6.66$ ,  $P=0.002$ ) as two-way interaction terms and STRAIN×SHOCK×HEAT ( $F_{(2,84)}=1.75$ ,  $P=0.18$ ) as the three-way interaction term. P values from subsequent planned comparisons are summarized in the Table. The six planned comparisons were judged significant if  $P\leq 0.01$ .

G. Seven-day memory after spaced training in 17-2 flies (FIG. 11): PIs from two strains (Can-S and 17-2) and two heat-shock regimens [-hs and +hs(3 hr)] were subjected to a TWO-WAY ANOVA with STRAIN ( $F_{(1,20)}=6.09$ ;  $P=0.02$ ) and HEAT-shock regimen ( $F_{(1,20)}=16.30$ ,  $P=0.001$ ) as main effects and STRAIN×TRAIN ( $F_{(1,20)}=7.73$ ,  $P=0.01$ ) as the interaction term. P values from subsequent planned comparisons are summarized in FIG. 11. The three planned comparisons were judged significant if  $P\leq 0.02$ .

H. One-day memory after spaced training in rsh; 17-2 double mutants (FIG. 12): PIs from three strains (17-2, rsh and rsh; 17-2) and two heat-shock regimens [-hs and +hs (3 hr)] were subjected to a TWO WAY ANOVA with STRAIN ( $F_{(2,30)}=32.05$ ;  $P<0.001$ ) and HEAT-shock regimen ( $F_{(1,30)}=59.68$ ,  $P<0.001$ ) as main effects and STRAIN×TRAIN ( $F_{(2,30)}=11.59$ ,  $P<0.001$ ) as the interaction term. P values from subsequent planned comparisons are summarized in FIG. 12. The five planned comparisons were judged significant if  $P\leq 0.01$ .

I. Learning after one-cycle training in rsh; 17-2 mutants (see text): PIs from these two strains (Can-S and rsh; 17-2) and two heat-shock regimens [-hs and +hs (3 hr)] were subjected to a TWO-WAY ANOVA with STRAIN ( $F_{(1,20)}=86.85$ ,  $P<0.001$ ) and HEAT-shock regimen ( $F_{(1,20)}=0.02$ ,  $P<0.89$ ) as main effects and STRAIN×HEAT ( $F_{(1,20)}=0.86$ ,  $P=0.37$ ) as the interaction term. P values from subsequent planned comparisons are summarized in the Table. The two planned comparisons were significant if  $P\leq 0.03$ .

J. Olfactory acuity in rsh; 17-2 flies (Table): PIs from these two strains (Can-S and rsh; 17-2), four different odor-levels ( $OCT-10^0$ ,  $OCT-10^{-2}$ ,  $MCH-10^0$  and  $MCH-10^{-2}$ ) and two heat-shock regimens [-hs, and +hs (3 hr)] were subjected to a THREE-WAY ANOVA with STRAIN ( $F_{(1,112)}=0.02$ ,  $P=0.88$ ), ODOR-level ( $F_{(3,112)}=50.03$ ,  $P<0.001$ ) and HEAT-shock regimen ( $F_{(1,112)}=29.86$ ,  $P<0.001$ ) as main effects, STRAIN×ODOR ( $F_{(3,112)}=2.15$ ,  $P=0.10$ ), STRAIN×HEAT ( $F_{(1,112)}=0.34$ ,  $P=0.56$ ) and ODOR×HEAT ( $F_{(3,12)}=6.41$ ,  $P=0.001$ ) as two-way interaction terms and STRAIN×ODOR×HEAT ( $F_{(3,112)}=1.12$ ,  $P=0.35$ ) as the three-way interaction term. P values from subsequent planned comparisons are summarized in the Table. The eight planned comparisons were judged significant if  $P\leq 0.01$ .

K. Shock reactivity in rsh; 17-2 flies (Table): PIs from these two strains (Can-S and rsh; 17-2), two shock groups (60V and

20V) and two heat-shock regimens [-hs and +hs (3 hr)] were subjected to THREE-WAY ANOVA with STRAIN ( $F_{(1,56)}=0.51$ ,  $P=0.48$ ), SHOCK ( $F_{(1,56)}=88.14$ ,  $P<0.001$ ) and HEAT-shock regimen ( $F_{(1,56)}=0.08$ ,  $P=0.77$ ) as main effects, STRAIN×SHOCK ( $F_{(1,56)}=0.12$ ,  $P=0.73$ ), STRAIN×HEAT ( $F_{(1,56)}=0.03$ ,  $P=0.86$ ) and SHOCK×HEAT ( $F_{(1,56)}=0.39$ ,  $P=0.53$ ) as two-way interaction terms and STRAIN×SHOCK×HEAT ( $F_{(1,84)}=1.58$ ,  $P=0.21$ ) as the three-way interaction term. P values from subsequent planned comparisons are summarized in the Table. The four planned comparisons were judged significant if  $P\leq 0.01$ .

#### Northern Analysis

15 For RNA collection, the heat-shock regimen was the same as for behavioral experiments. For any indicated time interval between heat-shock and collection, flies rested in food-containing vials at 25° C. Flies were collected and quickly frozen in liquid nitrogen. All Northern analyses used head RNA. The tube of frozen flies was repeatedly rapped sharply on a hard surface, causing the heads to fall off. The detached frozen heads were recovered by sieving on dry ice. Approximately 1000 heads were pooled for RNA preparation. Wild-type and transgenic flies for each individual time point always were processed in parallel. Flies that were not induced were handled in a similar manner to induced flies, except that the vials were not placed at 37° C. Total head RNA was isolated from each group of flies, and poly A+ RNA was isolated using oligo dT columns according to the instructions of the manufacturer (5'--->3' Inc.). The concentration of poly A+ mRNA was measured spectrophotometrically, and 0.5 mg of mRNA per lane was loaded and run on 1.2% formaldehyde-agarose gels. Northern blots were prepared, probed and washed (0.1× SSC at 65° C.) as described (Ausubel, F., *Current Protocols in Molecular Biology*, John Wiley and Sons, New York, 1994). For detection of the transgene, an 843 bp dCREB2-b cDNA fragment was subcloned into pKS+ and used to generate a uniformly-labeled antisense riboprobe. This fragment codes for the carboxyl-terminal 86 amino acids of the dCREB2-b protein plus 3' untranslated mRNA.

#### Western Blot Analysis and Antiserum

Western blot analysis was performed using a rabbit antiserum raised against a peptide corresponding to 16 amino acids in the basic region of the dCREB2-b cDNA with an additional COOH terminal Cys. The sequence of the peptide was: (SEQ ID NO.: 24) NH<sub>2</sub>-RKREIRLQKNREAAREC-COOH. The peptide was synthesized and coupled to Sulfo-SMCC (Pierce) activated keyhole limpet hemocyanin. The antigen was injected into rabbits (100 µg) and boosted at two week intervals. Sera was bled and tested for immune reactivity towards bacterially-expressed dCREB2-b protein. The antiserum was passed through a CM Affi-gel Blue column (Bio-rad), and the flow-through was concentrated by ammonium sulfate precipitation, resuspended and dialyzed against PBS (0.14 M NaCl, 2.7 mM KCl, 4.3 mM Na<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O, 1.4 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.3). The dialyzed serum was affinity-purified using a peptide column made using an Ag/Ab Immobilization kit (Immunopure from Pierce). After the antiserum was eluted using a 4 M MgCl<sub>2</sub>, 0.1 M HEPES pH 6.0 buffer, it was dialyzed into PBS and frozen.

Each data point represents approximately 5 fly heads. Groups of about 25-50 flies were collected and quickly frozen on liquid nitrogen until all of the time points had been collected. Heads were isolated resuspended in approximately 200 µl of 1× Laemmli sample buffer, allowed to thaw and

homogenized with a Dounce type B pestle. Samples were boiled for 5 minutes, and centrifuged for 10 minutes at room temperature in an Eppendoff microcentrifuge. The supernatants were collected and boiled again just prior to loading onto protein gels. Standard procedures were used to separate equal amounts of proteins from each sample on 12% polyacrylamide-SDS gels and to transfer them to PVDF membranes by electroblotting (Ausubel, F., *Current Protocols in Molecular Biology*, John Wiley and Sons, New York, 1994).

The membranes were blocked for 60 minutes with a 5% BSA solution made up in TBST (10 mM Tris, pH 7.9, 150 mM NaCl, 0.05% Tween 20). The primary antibody was diluted 1:1000 in TBST and incubated with the filter for 30 minutes. The membranes were washed three times with TBST for 5 minutes each time and then incubated for 30 minutes with an alkaline phosphatase-conjugated anti-rabbit IgG second antibody (Promega) diluted 1:7500 in TBST. The membranes were washed three more times as before and developed using a chromogenic alkaline phosphatase reaction according to manufacturers suggestions (Promega).

### Example 3

#### Transgene Expression Increased After Heat-Shock Induction

In order to interpret the effects of transgene induction on behavior, dCREB2-b expression in transgenic flies (17-2) after heat-shock induction was measured. Northern blot analysis revealed elevated levels of hs-dCREB2-b message in the 17-2 flies immediately and three hours after heat-shock (FIG. 7A). This induction was also detectable in brain cells using in situ hybridization. Western blot showed increased dCREB2-b protein immediately after induction (FIG. 7B). Elevated levels of the dCREB2-b protein were seen nine hours later and were still detectable twenty four hours after induction. These data indicate that increased amounts of dCREB2-b existed in brain cells throughout spaced training, which ended about six hours after heat induction.

The behavioral experiments also used transgenic flies (A2-2) which expressed a mutated dCREB2-b protein (dCREB2-mLZ). These mutations changed the two internal leucine residues of the leucine zipper to valine residues, and these changes have been shown to result in a protein which is unable to form dimers (Dwarki, V. J. et al., *EMBO J.*, 9: 225-232 (1990)). In transient co-transfection assays, the mutant protein was unable to block PKA-dependent transcription mediated by dCREB2-a, while the wild-type protein had blocking function. Western blot analysis showed that the wild-type and mutant blocker are expressed at similar levels beginning immediately after heat-shock induction and lasting for at least 6 hours (FIG. 7C). Therefore, it is unlikely that these two proteins have large differences in expression levels or stability in the transgenic flies.

Northern blot analysis of two different housekeeping genes, myosin light chain (Parker, V. P., et al., *Mol. Cell Biol.*, 5: 3058-3068 (1985)) and elongation factor  $\alpha$  (Hovemann, B., et al., *Nucleic Acids Res.*, 16: 3175-3194 (1988)), showed that steady-state levels of their RNAs were unaffected after transgene induction for at least 3 hours. Gel shift analysis using two different consensus DNA binding sites showed that there was no large effect on the gel shift species which formed after transgene induction for at least 9 hours. Cotransfection of the blocker did not interfere with the activity of a transcription factor from a different family in cell culture. Considered together, hs-dCREB2-b probably had fairly specific molecular modes of action after induction.

#### Assessment of the Role of CREBs in Long-Term Memory Formation

Flies were fed 35 mM cycloheximide (CXM) for 12-14 hours before, or for the 24-hr retention interval immediately after, massed or spaced training (FIG. 8). Each of these CXM feeding regimens significantly reduced one-day memory after spaced training but had no effect on one-day memory after massed training (FIG. 8). Thus, cycloheximide feeding immediately before or after spaced training disrupts one-day memory. These results suggest that protein synthesis is required soon after training for the formation of long-lasting memory.

The results in FIG. 8 show that cycloheximide feeding affects one-day retention after spaced training but not massed training. Different groups of wild-type (Can-S) flies were fed 5% glucose solution alone (hatched bars) or laced with 35 mM CXM (striped bars) either for 12-14 hr overnight before massed or spaced training or for the 24-hr retention interval immediately after training. One-day memory retention was significantly lower than normal in flies fed CXM before ( $P < 0.001$ ) or after ( $P < 0.001$ ) spaced training. In both cases, one-day retention in CXM-fed flies was reduced to a level similar to one-day memory after massed training in glucose-fed flies ( $P = 0.88$  for CXM before training and  $P = 0.71$  for CXM after training). In contrast, no difference was detected between CXM-fed and control flies for one-day memory after massed training ( $P = 0.49$  and  $P = 0.46$ , respectively).

One day retention after spaced training was unaffected in uninduced (-hs) transgenic flies (17-2) but was significantly reduced in induced (+hs) transgenic flies (FIG. 9A). In contrast, one-day retention after massed training was normal in both uninduced and induced transgenic flies (FIG. 9A). Comparisons of one-day retention after spaced or massed training between wild-type flies with (+hs) or without (-hs) heat-shock indicated that the heat-shock regimen itself did not have a non-specific effect on memory after either training protocol. Thus induction of the dCREB2-b transgene only affected (i.e., disrupted) one-day memory after spaced training.

One day retention after spaced or massed training in M11-1, a second line carrying an independent hs-dCREB2-b insertion, also was tested. Results with M11-1 were similar to those obtained with 17-2 (FIG. 9B). These results show that the effect of induced hs-dCREB2-b does not depend on any particular insertion site of the transgene.

The results in FIGS. 9A-9C show that induction of the dCREB2-b transgene disrupts one-day memory after spaced training, while one-day memory after massed training and learning are normal.

In FIG. 9A, different groups of wild-type (Can-S) flies (hatched bars) or hs-dCREB2-b transgenic (17-2) flies (striped bars) were given spaced training or massed training in the absence of heat shock (-hs) or three hours after heat shock (+hs). After training, flies were transferred to standard food vials and stored at 18° C. until one-day memory was assayed. No differences in one-day memory after spaced or massed training were detected between Can-S vs. 17-2 flies in the absence of heat shock (-hs;  $P = 0.83$  and  $0.63$ , respectively). When flies were trained three hours after heat shock (+hs), however, one-day memory was significantly different between Can-S vs. 17-2 flies after spaced training ( $P < 0.001$ ) but not after massed training ( $P = 0.23$ ). In fact, the one-day memory after spaced training was no different than that after massed training in induced 17-2 flies ( $P = 0.59$ ). In addition,

the heat-shock regimen did not produce a non-specific effect on one-day retention after spaced ( $P=0.59$ ) or massed ( $P=1.00$ ) training in Can-S flies.  $N=6$  performance indices (PIs) per group.

The experiment described in FIG. 9A was repeated in FIG. 9B with a second, independently derived dCREB2-b transgenic line, M11-1 (striped bars). Here again, a) no differences in one-day memory after spaced or massed training were detected between Can-S vs. M11-1 flies in the absence of heat-shock ( $-hs$ ;  $P=0.83$  and  $0.86$ , respectively), b) a significant difference between Can-S v. M11-1 for one-day memory after spaced training ( $P<0.001$ ) but not after massed training ( $P=0.85$ ) when trained three hours after heat-shock ( $+hs$ ), c) one-day memory after spaced training was no different than that after massed training in induced M11-1 flies ( $P=0.43$ ) and d) the heat-shock regimen did not produce a non-specific effect on one-day retention after spaced ( $P=0.59$ ) or massed ( $P=0.94$ ) training in Can-S flies.  $N=6$  PIs per group.

If induction of the transgene specifically affected LTM via disruption of gene expression, then learning should not be affected, since it does not require new protein synthesis. Different groups of flies were trained using one-cycle training either without heat-shock, or three or twenty four hours after heat-shock. These time points after induction were selected to correspond to the times when flies were trained and tested in the previous experiments (see FIGS. 9A and 9B). Induction of the transgene (dCREB2-b) in the 17-2 line had no effect on learning in either case (FIG. 9C).

In FIG. 9C, different groups of Can-S flies (hatched bars) or 17-2 transgenic flies (striped bars) received one-cycle training in the absence of heat shock ( $-hs$ ) or three ( $+hs$  3 hr) or 24 ( $+hs$  24 hr) hours after heat-shock and then were tested immediately afterwards. In each case, no differences between Can-S vs. 17-2 flies were detected ( $P_s=0.28$ ,  $0.64$  and  $0.42$ , respectively), indicating that learning was normal in induced or uninduced transgenic flies.  $N=6$  PIs per group.

Induction of the transgene which contained the mutant blocker (A2-2) did not affect one-day retention after spaced training, while the wild-type blocker (17-2) had a dramatic effect (FIG. 10). The w(iso CJ1) flies, whose one-day retention also was unaffected by heat induction, is the isogenic control for the mutant blocker transgenic flies. Since Western blot analysis showed that wild-type and mutant blockers probably have similar expression levels, this result suggests that the blocker requires an intact leucine zipper to function effectively.

FIG. 10 shows that induction of the hs-dCREB2-mLZ mutant blocker does not affect one-day retention after spaced training. Different groups of wild-type [w(isoCJ1)], hs-dCREB2-b transgenic (17-2) or mutant hs-dCREB2-mLZ transgenic flies (A2-2) received spaced training in the absence of heat-shock ( $-hs$ ) or three hours after heat-shock ( $+hs$ ). The flies were then handled and tested as in FIG. 9A. No differences in one-day memory after spaced training were detected between w(isoCJ1) vs. 17-2 flies or between w(isoCJ1) vs. A2-2 flies in the absence of heat shock ( $-hs$ ;  $P=0.38$  and  $0.59$ , respectively). When flies were trained three hours after heat shock ( $+hs$ ), however, one-day memory after spaced training was significantly different between w(isoCJ1) vs. 17-2 flies ( $P<0.001$ )—as in FIG. 9A—but was not different between w(isoCJ1) vs. A2-2 flies ( $P=0.78$ ). In addition, the heat-shock regimen did not produce a non-specific effect on one-day retention after spaced training in w(isoCJ1) or A2-2 flies ( $P=0.40$  and  $P=0.97$ , respectively).  $N=6$  performance indices (PIs) per group.

Olfactory acuity and shock reactivity are component behaviors essential for flies to properly learn odor-shock

associations. The Table shows the scores for these peripheral behaviors for Can-S versus 17-2 flies. With or without heat-shock, olfactory acuity and shock reactivity were normal in 17-2 transgenic flies. Thus, hs-dCREB2-b induction does not affect olfactory acuity or shock reactivity.

If induction of hs-dCREB2-b blocks long-term memory (LTM), then long-lasting memory also should be blocked. In wild-type flies, seven-day retention after spaced training consists solely of the CXM-sensitive LTM because the CXM insensitive ARM component has decayed away. In uninduced transgenic flies (17-2), seven-day retention after spaced training was similar to retention in uninduced wild-type flies ( $P=0.83$ ; FIG. 11). Seven-day retention was severely disrupted, however, in transgenic flies which were trained three hours after heat-shock ( $P=0.001$ ) and did not differ from zero ( $P=0.91$ ). In contrast, the heat-shock protocol had no detectable effect on seven-day memory in wild-type flies ( $P=0.39$ ). Thus, induction of hs-dCREB2-b disrupts long-term memory (LTM).

FIG. 11 shows that induction of hs-dCREB2-b completely abolishes 7-day memory retention. Previous analyses of radish mutants indicated that memory retention four or more days after spaced training reflects the sole presence of LTM. Thus, the effect of induced hs-dCREB2-b on LTM was verified by comparing 7-day retention after spaced training in Can-S (hatched bars) vs. 17-2 transgenic (striped bars) flies that were trained in the absence of heat-shock ( $-hs$ ) or three hours after heat shock ( $+hs$ ). Flies were stored in standard food vials at  $18^\circ$  C. during the retention interval.  $N=6$  PIs per group. Seven-day retention after spaced training did not differ between Can S and 17-2 in the absence of heat-shock ( $P=0.83$ ) but was significantly lower than normal in 17-2 flies after heat-shock ( $P=0.002$ ). In fact, 7-day retention after spaced training in induced 17-2 transgenic flies was not different from zero ( $P=0.92$ ). In addition, the heat-shock regimen did not affect 7-day retention after spaced training non-specifically in Can-S flies ( $P=0.39$ ).

If induction of the hs-dCREB2-b transgene specifically blocks LTM, then it should only affect the CXM-sensitive component of consolidated memory after spaced training. For both transgenic lines, 17-2 and M11-1, the effect of transgene induction looked similar to the effect that CXM had on wild-type flies (compare FIG. 8 with FIGS. 9A and 9B). This similarity suggested that the induced dCREB2-b protein completely blocked CXM-sensitive memory, leaving ARM intact. The radish mutation disrupts ARM (Folkers, E., et al., *Proc. Natl. Acad. Sci. USA*, 90: 8123-8127 (1993)), leaving only LTM one day after spaced training. Thus, a radish hs-dCREB2-b “double mutant” (rsh; 17-2) was constructed to allow examination of LTM in the absence of ARM. In the absence of heat-shock, rsh; 17-2 double-mutants and radish single-gene mutants yielded equivalent one-day retention after spaced training (FIG. 12). In contrast, when these flies were heat-shocked three hours before spaced training, one-day retention was undetectable in rsh; 17-2 flies but remained at mutant levels in radish flies. The double mutant also showed normal (radish-like) learning ( $P=0.59$ ) and normal (wild-type) olfactory acuity and shock reactivity in the absence of heat-shock versus three hours after heat shock (see the Table).

FIG. 12 shows that induction of hs-dCREB2-b completely abolishes one-day memory after spaced training in radish; 17-2 “double mutants.” Since radish is known to disrupt ARM, a clear view of the effect of hs-dCREB2-b on LTM was obtained in radish; 17-2 flies. One-day retention after spaced training was assayed in rsh; 17-2 double mutants and in 17-2 and rsh single-gene mutants as controls. Flies were trained in

the absence of heat-shock (hatched bars) or three hours after heat-shock (striped bars) and stored at 18° C. during the retention interval. As usual, induction of hs-dCREB2-b produced significantly lower one-day memory after spaced training in 17-2-flies ( $P < 0.001$ ). The heat-shock regimen, however, had no effect on such memory in radish mutants ( $P = 0.52$ ), which reflects only the presence of LTM. In contrast, heat-shock produced significantly lower scores in rsh; 17-2 double mutants ( $P < 0.001$ ), which were not different from zero ( $P = 0.20$ ). N=6 PIs per group.

TABLE

		Olfactory Acuity				Shock	
		OCT		MCH		Reactivity	
Heat Shock	Group	10 <sup>0*</sup>	10 <sup>-2</sup>	10 <sup>0*</sup>	10 <sup>-2</sup>	60 V	20 V
-hs	CAN-S	58 ± 3	32 ± 3	80 ± 2	33 ± 7	79 ± 5	52 ± 5
	17-2	60 ± 3	34 ± 8	77 ± 3	37 ± 5	87 ± 3	43 ± 2
+hs (3 hrs)	CAN-S	69 ± 4	41 ± 4	77 ± 2	25 ± 9	74 ± 5	58 ± 6
	17-2	71 ± 4	37 ± 3	76 ± 5	26 ± 3	78 ± 3	67 ± 5
+hs (24 hr)	CAN-S	66 ± 2	56 ± 8	79 ± 4	33 ± 2	84 ± 3	63 ± 3
	17-2	65 ± 3	42 ± 6	76 ± 3	41 ± 5	85 ± 2	60 ± 6
-hs	CAN-S	51 ± 4	39 ± 5	72 ± 5	33 ± 7	87 ± 3	52 ± 5
	rsh; 17-2	57 ± 3	39 ± 5	74 ± 5	29 ± 4	82 ± 4	53 ± 6
+hs (3 hr)	CAN-S	72 ± 4	48 ± 3	66 ± 2	60 ± 3	80 ± 4	58 ± 6
	rsh; 17-2	68 ± 4	46 ± 6	78 ± 2	49 ± 4	83 ± 1	50 ± 5

\*Olfactory acuity and shock reactivity were assayed in untrained flies with the methods of Boynton, S. and T. Tully, *Genetics*, 131: 655-672 (1992) and Dura, J-M., et al., *J. Neurogenet.*, 9: 1-14 (1993), respectively (see Examples for more details). N = 98 PIs per group. Planned comparisons between Can-S vs. mutant flies failed to detect any significant differences with any heat-shock regimen.

\*10<sup>0</sup> is manual concentration and corresponds to 10<sup>-3</sup> for bubbler.

The following materials and methods were used in the work described in Examples 5 and 6.

#### Pavlovian Learning and Memory and Testing

During one training session, a group of about 100 flies was exposed sequentially to two odors [either octanol (OCT) or methylcyclohexanol (MCH)] for 60 seconds with 45-second rest intervals after each odor presentation. During exposure to the first odor, flies received twelve 1.5-second pulses of 60 V DC with a 5-second interpulse interval.

After training, flies were transferred to food vials and stored at 18° C. for a seven-day retention interval. Conditioned odor-avoidance responses then were tested by transferring flies to the choice point of a T-maze, where they were exposed simultaneously to OCT and MCH carried on converging currents of air in the distal ends of the T-maze arms and out the choice point.

Flies were allowed to distribute themselves in the T-maze arms for 120s, after which they were trapped in their respective arms, anesthetized and counted. The “percent correct” then was calculated as the number of flies avoiding the shock-paired odor (they were in the opposite T-maze arm) divided by the total number of flies in both arms. (The number of flies left at the choice point, which usually was less than 5%, were not included in this calculation.) Finally, a performance index (PI) was calculated by averaging the percent corrects of two reciprocal groups of flies—one where OCT and shock were paired, the other where MCH and shock were paired—and then by normalizing the average so that a PI=0 represented a 50:50 distribution in the T-maze and a PI=100 represented 100% avoidance of the shock-paired odor.

All behavioral experiments were designed in a balanced fashion with N=2 PIs per group collected per day; then replicated across days to generate final Ns. In all experiments, the experimenter was blind to genotype.

#### Statistical Analyses of Behavior Data

PIs are distributed normally (Tully, T. and D. Gold, *J. Neurogenet.*, 9: 55-71 (1993)). Consequently, untransformed (raw) data were analyzed parametrically with JMP3.01 sta-

tistical software (SAS Institute Inc., Cary N.C.). Negative accelerating exponential Gompertz (growth) functions (see Lewis, D., *Quantitative Methods in Psychology*, McGraw-Hill, New York, N.Y. (1960)) were fit to the data in FIGS. 13A and 13B via nonlinear least squares with iteration.

#### Example 5

##### Effect on Long Term Memory of Repeated Training Sessions

Seven-day memory retention (a measure of long term memory) in wild-type (Can-S) flies is induced incrementally by repeated training sessions. An automated version of a discriminative classical conditioning procedure was used to electroshock flies during exposure to one odor (CS+) but not to a second odor (CS-). Seven days after one or more training sessions, memory retention of conditioned odor avoidance responses was quantified in a T-maze, where flies were presented the CS+ and CS-simultaneously for 120 seconds.

In FIG. 13A, long term memory as a function of the number of training sessions is indicated by open circles. One training session produced a mean performance index (PI±SEM) near zero. Additional training sessions with a 15-minute rest interval between each, however, yielded a steady increase in mean PIs with a maximum of 39 after ten training sessions. Ten additional training sessions produced similar performance. A nonlinear “growth” function (solid line) was fit to the individual PIs using an iterative least squares method. N=13, 6, 6, 6, 13, 7, 7, 7, 7, 6, 7 and 7 PIs for groups receiving 1-10, 15 and 20 training sessions, respectively.

#### Effect on Long Term Memory of the Rest Interval between Each Training Session

Seven-day memory retention (a measure of long term memory) in wild-type (Can-S) flies is induced incrementally by the rest interval between each training session. As described in Example 5, an automated version of a discriminative classical conditioning procedure was used to electroshock flies during exposure to one odor (CS+) but not to a second odor (CS-). Seven days after one or more training sessions, memory retention of conditioned odor avoidance responses was quantified in a T-maze, where flies were presented the CS+ and CS- simultaneously for 120 seconds.

In FIG. 13B, long term memory as a function of the rest interval is indicated by open circles. Ten training sessions with no rest interval between each (massed training) produced a mean PI near zero. Increasing the rest interval between each of ten training sessions yielded a steady increase in mean PIs with a maximum of 34 for a 10-minute rest interval. Rest intervals up to ten minutes longer produced similar performance. A nonlinear growth function (solid line) was fit to the data as above. N=12, 6, 6, 6, 6, 13, 7, 7, 7, 7, 7 and 7 PIs for groups receiving 0-10, 15 and 20 minutes of rest between each training session.

The following materials and methods were used in the work described in Examples 7-10.

#### Isolating Transgenic Flies

EcoRI restriction sites were added (using PCR) just 5' to the putative translation initiation site and just 3' to the translation termination site in the dCREB2-a cDNA. This fragment was sequenced and subcloned into CaSpeR hs43, a mini-white transformation vector which contains the hsp70 promoter, in the orientation so that the dCREB2-a open reading frame is regulated by the hsp70 promoter. Germ-line transformation was accomplished by injecting into isogenic w(isoCJ1) embryos using standard techniques (Spradling, A. C. and G. M. Rubin, *Science*, 218: 341-347 (1982); Rubin, G. M. and A. Spradling, *Science*, 218: 348-353 (1982)). By injecting DNA into the relatively homogeneous genetic background of w(isoCJ1), outcrossing of the resulting germ-line transformants to equilibrate (heterogeneous) genetic backgrounds was not necessary. Two transgenic lines, C28 and C30, each with one independent P-element insertion were generated and characterized. They appeared normal in general appearance, fertility and viability. Flies homozygous for the C28 or C30 transgene were bred and used for all experiments.

#### Heat Shock Regimen

For heat-shock induction, flies were collected within two days of eclosion, placed in glass bottles in groups of about 600, and incubated overnight at 25° C. and 70% relative humidity. The next day, three hours before training, groups of approximately 100 flies were transferred to foam-stoppered glass shell vials containing a strip of filter paper to absorb excess moisture. The vials then were submerged in a 37° C. water bath until the bottom of the foam stopper (inside the vial) was below the surface of the water, thereby insuring that the flies could not escape heat-shock. The vial remained submerged for 30 minutes, after which the flies were transferred to a standard food vial for a 3-hr recovery period at 25° C. and 70% relative humidity. Training began immediately after the recovery period.

PIs from the three strains (Can-S, C28 and C30) and six training-regiments (1×+hs, 2×massed+hs, 10× massed +hs, 1×-hs, 2× massed -hs and 10× massed -hs) were subjected to a TWO-WAY ANOVA with STRAIN ( $F_{(2,102)}=48.34$ ;  $P<0.001$ ) and TRAINING-regimen ( $F_{(5,102)}=25.47$ ,  $P<0.001$ ) as main effects and STRAIN×TRAIN ( $F_{(10,102)}=5.85$ ,  $P<0.001$ ) as the interaction term. Since preliminary experiments preceded all of the experiments summarized herein, all pairwise comparisons were planned. To maintain an experimentwise error rate of  $\alpha=0.05$ , the individual comparisons summarized in FIG. 15B were judged significant if  $P<0.002$  (Sokal, R. R. and F. J. Rohlf, *Biometry*, 2nd Edition, W.H. Freeman and Company, New York (1981)).

#### Example 7

#### A Molecular Switch for the Formation of Long Term Memory

FIG. 14 presents a conceptual method of a molecular switch for the formation of LTM, based on differential regulation of CREB isoforms with opposing functions.

Immediately after one training session, the relevant CREB activators and repressors are induced. Their combined functions (rather than molecular concentrations) are equivalent and yielded no net effect of CREB activators. Thus, repeated sessions of massed training (no rest interval) never induce LTM (see FIG. 15A). CREB repressors functionally inactivate faster than CREB activators, however, yielding an increasing net effect of CREB activators ( $\Delta C$ ) with time (see FIG. 13B). If  $\Delta C$  is positive at the end of a particular rest interval during spaced training, then CREB activators are free to initiate downstream events involved with the formation of LTM. Usually,  $\Delta C$  after one training session is not large enough to yield much LTM. Thus, repeated spaced training sessions serve to increase  $\Delta C$  incrementally eventually to produce maximal LTM (see FIG. 13A).

Experimental verification of two predictions from this model involving CREB as a molecular switch for long term memory formation is shown in FIGS. 15A-15C and discussed in Examples 8-10.

#### Example 8

#### Effect on Long Term Memory of Having Equal Amounts of CREB Activators and Repressors Immediately After One Training Session

The model of a molecular switch for LTM predicts that the functional effects of all CREB activators and repressors are equal immediately after one training session ( $\Delta C=0$ ). If no rest interval occurred between additional training sessions (massed training), then functional CREB activator would not accumulate, thereby preventing the induction of downstream events required for LTM induction.

To test this notion, wild-type (Can-S) flies were subjected to 48, instead of the usual 10 (see FIG. 15B), massed training sessions (48× massed) or, as a positive control, to 10 spaced training sessions with a 15-minute rest interval (10× spaced). Seven-day memory after such massed training was near zero (FIG. 15A), while that after spaced training was near its usual maximum value (see FIG. 13A). Thus, nearly five times the usual amount of massed training still did not induce LTM. N=6 PIs for each group.



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PIs from two groups (10× spaced or 48× massed) of wild-type (Can-S) flies were subjected to a ONE-WAY ANOVA with GROUP ( $F_{[1,10]}=51.13$ ;  $P<0.001$ ) as the main effect. A subsequent planned comparison revealed that the mean PI of the 48× massed group did not differ significantly from zero ( $t_{[1,10]}=1.66$ ;  $P=0.127$ ).

## Example 9

## Effect on Long Term Memory of Increasing Amounts of CREB Activator

The model of a molecular switch for LTM predicts that experimentally increasing the amount of CREB activator will eliminate the requirements for at least 10 repeated training sessions with a 10-minute rest interval between each to produce maximal LTM.

To test this idea, two transgenic lines (C28 and C30) carrying an inducible hsp-dCREB2-a activator construct inserted into different cytological locations were generated. Different groups of flies from these two transgenic lines were subjected, along with wild-type (Can-S) flies, to 1 (1×), 2 (2×) or (10×) massed training sessions three hours after heat-shock induction of the transgene (induced) or in the absence of heat-shock (uninduced).

Without heat-shock, seven-day memory in all three strains did not differ from zero after one, two or ten massed training sessions (all  $P_s>0.002$ ). With heat-shock, seven-day memory in wild-type flies remained near zero in each massed training group (all  $P_s>0.002$ ). In contrast, seven-day memory was significant (near the maximum of 35) after ten massed sessions in both the C28 and C30 transgenic lines (all  $P_s<0.0001$ ). Moreover, seven-day memory after one training session was similar to that after ten training sessions in both C28 ( $P=0.89$ ) and C30 ( $P=0.89$ ) transgenic flies. Thus, maximum LTM was induced after just one training session in transgenic flies expressing abnormally high levels of CREB activator.  $N=10$ , 4 and 6 PIs for each group of Can-S, C28 and C30, respectively.

## Example 10

## Olfactory Acuity and Shock Reactivity

Odor avoidance responses to OCT or to MCH were quantified with the method of Boynton, S. and T. Tully, *Genetics*, 131: 655-672 (1992), given a choice between an odor and air. The odors are naturally aversive, and flies usually chose air and avoided the T-maze arm containing the odor. After 120 seconds, the flies were trapped in their respective arms of the T-maze, anesthetized and counted. A PI was calculated as a normalized percent correctly avoiding the odor (cf. Example 5). PIs from these two strains and two odor-groups (OCT and MCH) were subjected to a TWO-WAY ANOVA with STRAIN ( $F_{(1,12)}=1.57$ ,  $P=0.23$ ) and ODOR ( $F_{(1,12)}=0.07$ ,  $P=0.80$ ) as main effects and DRUG×ODOR ( $F_{(1,12)}=0.15$ ,  $P=0.71$ ) as the interaction term. The two subsequent planned comparisons were judged significant if  $P<0.025$ .

Shock reactivity was quantified with the method of Dura, J-M., et al., *J. Neurogenet.*, 9: 1-14 (1993) in wild-type (Can-S) flies, or in a transgenic line (C28) carrying an inducible hsp-dCREB2-a construct, three hours after a 30-minute heat shock at 37° C. Briefly, flies were placed in a T-maze and given a choice between an electrified grid (60V DC) in one T-maze arm and an unconnected grid in the other. After 120 seconds, the flies were trapped in their respective T-maze arms, anesthetized and counted. A PI was calculated as for

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olfactory acuity. PIs from these two strains were subjected to a ONE-WAY ANOVA with STRAIN ( $F_{(1,6)}=13.03$ ,  $P=0.01$ ) as the main effect.

Naive avoidance responses to odors or to shock three hours after heat-shock did not differ between wild-type (Can-S) versus transgenic (C28) flies for the two odorants (MCH and OCT) used for conditioning experiments ( $P=0.27$ , 0.55, respectively).  $N=4$  PIs per group. Naive shock avoidance responses three hours after training for transgenic flies were slightly lower than those for wild-flies ( $P=0.01$ ).  $N=4$  PIs per group.

Examples 11-13 pertain to the *Drosophila* nitric oxide synthase work.

## Example 11

Low Stringency Hybridization to a Phage Library of the *Drosophila*Genome and Screening of *Drosophila* cDNA Library

$6 \times 10^4$  plaques of a genomic *Drosophila*  $\lambda$ DASH library with the 1.3 kb Bgl II fragment of rat neuronal NOS cDNA (residues 3282-4573) under low stringency conditions of 40% formamide were screened as described in W. M. McGinnis et al., *Nature*, 308: 428 (1984). Fifty positive phage were purified and grouped based on inter se hybridization. The contig containing the 2.4R fragment of dNOS was comprised of 15 phage clones. Regions of cross-hybridization to the rat probe were identified, subcloned and three of them were sequenced. The other two did not contain sequences homologous to any protein in the database. A *Drosophila* head cDNA library (a gift from P. Salvaterra) was screened with the 2.4R fragment isolated from phage clone  $\lambda$ 8.11 in standard conditions. All phage purification and cloning steps were done with standard methods (J. Sambrook, E. F. Fritsch, T. Maniatis, *Molecular Cloning, A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1989)). cDNA fragments were subcloned into Bluescript (Stratagene) and sequenced on both strands with Sequenase 2.0 (USB).

## Example 12

Activity of *Drosophila* Nitric Oxide Synthase (dNOS)

The expression construct for activity assays contained dNOS cDNA (with an XbaI site engineered immediately upstream of the ATG codon) cloned into the XbaI and SmaI sites of the pCGN expression vector (M. Tanaka and W. Herr, *Cell*, 60: 375 (1990)). 293 human kidney cells were transfected with 15  $\mu$ g of the dNOS construct, or vector DNA, and precipitated with calcium phosphate as described in (M. J. Imperiale, L. T. Feldman and J. R. Nevins, *Cell*, 35: 127 (1983)). Cells were collected 2 days later and protein extracts were prepared as described in (J. Sambrook, E. F. Fritsch, T. Maniatis, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1989)).

The fusion protein for raising anti-dNOS antibodies was made by cloning a 0.29 kb Eam11051-SacI fragment of dNOS cDNA (this fragment codes for 97 N-terminal amino acids of dNOS ORF) into EcoRI site of pGEX-KG (K. Guan and J. E. Dixon, *Anal. Biochem.*, 192: 262 (1991)). The fusion protein was expressed in BL21 *E. coli* strain and purified over Glutathione-Sepharose columns (Pharmacia) as described in G. J. Hannon, D. Demetrick and D. Beach, *Genes & Dev.*, 7: 2378 (1993). Immunization of rabbits, and serum prepara-

tion, was done by Hazleton Research Products, Inc. (Denver). The DNOS protein was detected on Western blots using a 1:500 dilution of rabbit serum, and cross-reacting bands were visualized with anti-rabbit alkaline phosphatase conjugate (Promega) according to the protocol provided.

The enzymatic assay was done essentially as described previously (D. Bredt and S. Snyder, *Proc. Natl. Acad. Sci. USA*, 87: 682 (1990)). A 100 ml reaction mixture containing 25  $\mu$ l (50-100  $\mu$ g) of soluble protein extract, 50 mM Hepes pH 7.4, 3>1M FAD, 3  $\mu$ M FMN, 10  $\mu$ M tetrahydrobiopterin (ICN), 1 mM DTT, 0.8 mM CaCl<sub>2</sub>, 1 mM NADPH, 10  $\mu$ g/ml calmodulin, 2  $\mu$ l of [<sup>3</sup>H]L-arginine (35.7 Ci/mmol, NEN) and 50 mM L-valine in was incubated for 60 minutes at 37° C. The reaction was stopped with 0.5 ml 20 mM Hepes pH 5.5, 2 mM EDTA, 2 mM EGTA, loaded on 0.5 ml Dowex AG 50WX-8 (Na<sup>+</sup> form) column and eluted with 3 $\times$ 0.5 ml of the stop buffer. Radioactivity present in the eluent was quantified in a scintillation counter.

FIGS. 17A-17B show the expression of DNOS enzymatic activity in 293 kidney cells. FIG. 17A shows the results of a Western blot analysis of protein extracts from 293 cells transfected with vector alone (lane 293+vector) or with dNOS cDNA construct (lane 293+dNOS). 25  $\mu$ g of soluble protein extracts was resolved on 7.5% polyacrylamide gel, transferred to nitrocellulose membrane and treated with anti-DNOS antibody. The arrow indicates the position of the DNOS protein. Positions of molecular weight markers (in kD) are shown on the left.

FIG. 17B shows significant DNOS enzyme activity measured in 293 cell extracts by conversion of <sup>3</sup>H-L-arginine to <sup>3</sup>H-L-citrulline. Enzymatic activity was detected only in cells transfected with dNOS cDNA construct (groups B-D) and is presented as specific activity (pmol of citrulline/mg/min.). The DNOS activity also was measured in the presence of 1 mM EGTA without exogenous Ca<sup>2+</sup> or calmodulin (group C), or in the presence of 100 mM L-NAME (group D). N=4 reactions per group.

### Example 13

#### Splicing Pattern of dNOS

Heads and bodies of adult flies were separated on sieves. Total RNA was isolated by the guanidinium isothiocyanate method (P. Chomczynski and N. Sacchi, *Anal. Biochem.*, 162: 156 (1987)). Poly(A)<sup>+</sup> RNA selection, Northern blot and hybridization were done with standard methods (J. Sam-

brook, E. F. Fritsch, T. Maniatis, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1989)). The blot was hybridized with random-primed dNOS cDNA (10<sup>6</sup> cpm/ml), washed in 0.1 $\times$ SSC and 0.1% SDS at 65° C. and exposed to X-ray film for 72 hours.

Two 25-mer primers (corresponding to residues 1374-1399 (the top primer) and 1793-1817 (the bottom primer) in the dNOS sequence) were used to amplify fragments of two dNOS splice products. Each RT-PCR reaction contained 30 ng of poly(A)<sup>+</sup> head RNA. In the first stage (RT), 90 ng of the bottom primer and 5U of rTth polymerase (Perkin-Elmers) were added and the mixture was incubated in the MJ Research Minicycler™ in the following sequence of conditions: 95° C./1 minute, 67° C./45 seconds, 70° C./13 minutes. The second stage (PCR) was carried out as follows: 94° C./45 seconds, 63° C./45 seconds, 70° C./90 seconds and was repeated for 35 cycles. Products of the reaction were analyzed on a denaturing polyacrylamide (8%) gel. Bands of interest were isolated, reamplified, cloned into pCR1000 (InVitrogen) and sequenced with Sequenase kit (USB).

Northern blot analysis of dNOS expression in adult flies shows a 5.0 kb dNOS transcript present in heads (FIG. 18A). Each lane contained 10 mg of poly(A)<sup>+</sup>mRNA isolated from *Drosophila* heads (H) or bodies (B). The Northern blot was hybridized with the dNOS cDNA as described above. Positions of size markers (in kb) are shown on the left. The blot was overprobed with myosin light chain (MLC) (Parker, V. P., *Mol. Cell Biol.*, 5: 3058-3068 (1985)) as a standard for RNA concentration.

FIG. 18B shows that the dNOS gene expresses two alternatively spliced mRNA species. RT-PCR reactions were performed on poly(A)<sup>+</sup> mRNA isolated from *Drosophila* heads and were resolved on 8% polyacrylamide gel. Arrows indicate the positions of DNA fragments of expected sizes: the 444 bp long-form fragment and the 129 bp short-form fragment (lane +RNA). Other bands present in this lane are artifacts from heteroduplexes that failed to denature. Poly(A)<sup>+</sup> mRNA was omitted from the control reaction (lane -RNA), which otherwise was done in identical conditions. Size markers (kb ladder) are shown in the middle lane (KB).

#### Equivalents

Those skilled in the art will know, or be able to ascertain, using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. These and all other equivalents are intended to be encompassed by the following claims.

#### SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 25

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<211> LENGTH: 1083

<212> TYPE: DNA

<213> ORGANISM: *Drosophila* sp.

<220> FEATURE:

<221> NAME/KEY: CDS

<222> LOCATION: (1)..(1080)

<400> SEQUENCE: 1

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1 5 10 15

48

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ggc tcc aat gac gtg gtc gat gtc gtt gcc caa cag gcg gcg gca gcg	96
Gly Ser Asn Asp Val Val Asp Val Val Ala Gln Gln Ala Ala Ala Ala	
20 25 30	
gtg ggc ggc ggc ggt gga gga gga gga ggc ggc ggc ggt ggt ggt aac	144
Val Gly Gly Gly Gly Gly Gly Gly Gly Gly Gly Gly Gly Gly Gly Asn	
35 40 45	
ccc cag cag cag caa cag aac cca caa agt aca acg gcc ggc ggt cca	192
Pro Gln Gln Gln Gln Gln Asn Pro Gln Ser Thr Thr Ala Gly Gly Pro	
50 55 60	
acg ggt gcg acg aac aac gcc cag gga ggc gga gtg tcc tcc gtg ctg	240
Thr Gly Ala Thr Asn Asn Ala Gln Gly Gly Gly Val Ser Ser Val Leu	
65 70 75 80	
acc acc acc gcc aac tgc aac ata caa tac ccc atc cag acg ctg gcg	288
Thr Thr Thr Ala Asn Cys Asn Ile Gln Tyr Pro Ile Gln Thr Leu Ala	
85 90 95	
cag cac gga ctg cag gtg agc att tgg gga ccg ggt gct tgg tgt caa	336
Gln His Gly Leu Gln Val Ser Ile Trp Gly Pro Gly Ala Trp Cys Gln	
100 105 110	
ctg tcg agt gtc agg tgt tac gga tcc cag cca gaa gtg gct acc aag	384
Leu Ser Ser Val Arg Cys Tyr Gly Ser Gln Pro Glu Val Ala Thr Lys	
115 120 125	
gat gtg cag tcc gtg ata cag gcc aat ccc tcg gga gtc ata cag aca	432
Asp Val Gln Ser Val Ile Gln Ala Asn Pro Ser Gly Val Ile Gln Thr	
130 135 140	
gca gct gga acc cag cag cag caa cag gcg ctg gcc gcc gcc aca gcg	480
Ala Ala Gly Thr Gln Gln Gln Gln Ala Leu Ala Ala Ala Thr Ala	
145 150 155 160	
atg cag aag gtg gtc tac gtg gcc aag ccg ccg aac tcg acg gtc atc	528
Met Gln Lys Val Val Tyr Val Ala Lys Pro Pro Asn Ser Thr Val Ile	
165 170 175	
cac acg acg cct ggc aat gca gtg caa gtg cgt aac aaa atc cct cca	576
His Thr Thr Pro Gly Asn Ala Val Gln Val Arg Asn Lys Ile Pro Pro	
180 185 190	
acc ttt cca tgt aag atc aag ccc gaa ccg aac acg cag cac ccg gag	624
Thr Phe Pro Cys Lys Ile Lys Pro Glu Pro Asn Thr Gln His Pro Glu	
195 200 205	
gac agc gac gag agt ctg tcg gac gac gat tcc cag cac cac cgc agc	672
Asp Ser Asp Glu Ser Leu Ser Asp Asp Asp Ser Gln His His Arg Ser	
210 215 220	
gag ctg acg cga cgg ccg tcg tac aat aag atc ttc acc gag atc agc	720
Glu Leu Thr Arg Arg Pro Ser Tyr Asn Lys Ile Phe Thr Glu Ile Ser	
225 230 235 240	
ggt ccg gac atg agc ggc gca tcg ctt ccc atg tcc gac ggc gtg ctc	768
Gly Pro Asp Met Ser Gly Ala Ser Leu Pro Met Ser Asp Gly Val Leu	
245 250 255	
aat tcc cag ctg gtg ggg acc gga gcg ggg ggc aat gcg gcg aac agc	816
Asn Ser Gln Leu Val Gly Thr Gly Ala Gly Gly Asn Ala Ala Asn Ser	
260 265 270	
tcc ctg atg caa ttg gat ccc acg tac tac ctg tcc aat cgg atg tcc	864
Ser Leu Met Gln Leu Asp Pro Thr Tyr Tyr Leu Ser Asn Arg Met Ser	
275 280 285	
tac aac acc aac aac agc ggg ata gcg gag gat cag acc cgt aag cgc	912
Tyr Asn Thr Asn Asn Ser Ser Ile Ala Glu Asp Gln Thr Arg Lys Arg	
290 295 300	
gag atc cgg ctg cag aag aac agg gag gcg gcg cgt gag tgc cgg cgc	960
Glu Ile Arg Leu Gln Lys Asn Arg Glu Ala Ala Arg Glu Cys Arg Arg	
305 310 315 320	
aag aag aag gag tac atc aag tgc ctg gag aat cga gtg gcg gtg cta	1008
Lys Lys Lys Glu Tyr Ile Lys Cys Leu Glu Asn Arg Val Ala Val Leu	
325 330 335	

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gag aac caa aac aaa gcg ctc atc gag gag ctg aag tcg ctc aag gag 1056  
 Glu Asn Gln Asn Lys Ala Leu Ile Glu Glu Leu Lys Ser Leu Lys Glu  
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ctc tat tgt cag acc aag aac gat tga 1083  
 Leu Tyr Cys Gln Thr Lys Asn Asp  
           355                  360

<210> SEQ ID NO 2  
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 <213> ORGANISM: Drosophila sp.

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                   20                  25                  30

Val Gly Gly Gly Gly Gly Gly Gly Gly Gly Gly Gly Gly Gly Gly Asn  
           35                  40                  45

Pro Gln Gln Gln Gln Gln Asn Pro Gln Ser Thr Thr Ala Gly Gly Pro  
   50                  55                  60

Thr Gly Ala Thr Asn Asn Ala Gln Gly Gly Gly Val Ser Ser Val Leu  
 65                  70                  75                  80

Thr Thr Thr Ala Asn Cys Asn Ile Gln Tyr Pro Ile Gln Thr Leu Ala  
                   85                  90                  95

Gln His Gly Leu Gln Val Ser Ile Trp Gly Pro Gly Ala Trp Cys Gln  
           100                  105                  110

Leu Ser Ser Val Arg Cys Tyr Gly Ser Gln Pro Glu Val Ala Thr Lys  
           115                  120                  125

Asp Val Gln Ser Val Ile Gln Ala Asn Pro Ser Gly Val Ile Gln Thr  
   130                  135                  140

Ala Ala Gly Thr Gln Gln Gln Gln Gln Ala Leu Ala Ala Ala Thr Ala  
 145                  150                  155                  160

Met Gln Lys Val Val Tyr Val Ala Lys Pro Pro Asn Ser Thr Val Ile  
           165                  170                  175

His Thr Thr Pro Gly Asn Ala Val Gln Val Arg Asn Lys Ile Pro Pro  
           180                  185                  190

Thr Phe Pro Cys Lys Ile Lys Pro Glu Pro Asn Thr Gln His Pro Glu  
   195                  200                  205

Asp Ser Asp Glu Ser Leu Ser Asp Asp Asp Ser Gln His His Arg Ser  
   210                  215                  220

Glu Leu Thr Arg Arg Pro Ser Tyr Asn Lys Ile Phe Thr Glu Ile Ser  
 225                  230                  235                  240

Gly Pro Asp Met Ser Gly Ala Ser Leu Pro Met Ser Asp Gly Val Leu  
           245                  250                  255

Asn Ser Gln Leu Val Gly Thr Gly Ala Gly Gly Asn Ala Ala Asn Ser  
           260                  265                  270

Ser Leu Met Gln Leu Asp Pro Thr Tyr Tyr Leu Ser Asn Arg Met Ser  
           275                  280                  285

Tyr Asn Thr Asn Asn Ser Gly Ile Ala Glu Asp Gln Thr Arg Lys Arg  
   290                  295                  300

Glu Ile Arg Leu Gln Lys Asn Arg Glu Ala Ala Arg Glu Cys Arg Arg  
 305                  310                  315                  320

Lys Lys Lys Glu Tyr Ile Lys Cys Leu Glu Asn Arg Val Ala Val Leu  
           325                  330                  335

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Glu Asn Gln Asn Lys Ala Leu Ile Glu Glu Leu Lys Ser Leu Lys Glu  
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Leu Tyr Cys Gln Thr Lys Asn Asp  
 355 360

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 <213> ORGANISM: Drosophila sp.

<400> SEQUENCE: 3

Arg Lys Arg Glu Ile Arg Leu Gln Lys Asn Arg Glu Ala Ala Arg Glu  
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Cys Arg Arg Lys Lys Lys Glu Tyr Ile Lys Cys Leu Glu Asn Arg Val  
 20 25 30

Ala Val Leu Glu Asn Gln Asn Lys Ala Leu Ile Glu Glu Leu Lys Ser  
 35 40 45

Leu Lys Glu Leu Tyr Cys  
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<210> SEQ ID NO 4  
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 <212> TYPE: PRT  
 <213> ORGANISM: Unknown  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Description of Unknown: Mammalian CREB  
 polypeptide

<400> SEQUENCE: 4

Arg Lys Arg Glu Val Arg Leu Met Lys Asn Arg Glu Ala Ala Arg Glu  
 1 5 10 15

Cys Arg Arg Lys Lys Lys Glu Tyr Val Lys Cys Leu Glu Asn Arg Val  
 20 25 30

Ala Val Leu Glu Asn Gln Asn Lys Thr Leu Ile Glu Glu Leu Lys Ala  
 35 40 45

Leu Lys Asp Leu Tyr Cys  
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<210> SEQ ID NO 5  
 <211> LENGTH: 54  
 <212> TYPE: PRT  
 <213> ORGANISM: Unknown  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Description of Unknown: Mammalian CREM  
 polypeptide

<400> SEQUENCE: 5

Arg Lys Arg Glu Leu Arg Leu Met Lys Asn Arg Glu Ala Ala Arg Glu  
 1 5 10 15

Cys Arg Arg Lys Lys Lys Glu Tyr Val Lys Cys Leu Glu Asn Arg Val  
 20 25 30

Ala Val Leu Glu Asn Gln Asn Lys Thr Leu Ile Glu Glu Leu Lys Ala  
 35 40 45

Leu Lys Asp Leu Tyr Cys  
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<210> SEQ ID NO 6  
 <211> LENGTH: 54  
 <212> TYPE: PRT  
 <213> ORGANISM: Unknown  
 <220> FEATURE:

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<223> OTHER INFORMATION: Description of Unknown: Mammalian ATF-1 polypeptide

<400> SEQUENCE: 6

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1 5 10 15

Cys Arg Arg Lys Lys Lys Glu Tyr Val Lys Cys Leu Glu Asn Arg Val  
20 25 30

Ala Val Leu Glu Asn Gln Asn Lys Thr Leu Ile Glu Glu Leu Lys Thr  
35 40 45

Leu Lys Asp Leu Tyr Ser  
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<211> LENGTH: 798

<212> TYPE: DNA

<213> ORGANISM: Drosophila sp.

<220> FEATURE:

<221> NAME/KEY: CDS

<222> LOCATION: (1)..(798)

<400> SEQUENCE: 7

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Met Leu Leu Gly Glu Asn Met Phe Ser Thr Phe Thr Ser Leu Asp Ala  
1 5 10 15

gct acc gct aca acc aac acc ggt gaa ttc tta atg aat gaa tct cca 96  
Ala Thr Ala Thr Thr Asn Thr Gly Glu Phe Leu Met Asn Glu Ser Pro  
20 25 30

agg caa gaa gcc ggt gac tta atg ttg gat agt ctg gat ttc aac att 144  
Arg Gln Glu Ala Gly Asp Leu Met Leu Asp Ser Leu Asp Phe Asn Ile  
35 40 45

atg ggc gaa aac ctg gca gat gat ttc cag acc tcg gct tca cca gct 192  
Met Gly Glu Asn Leu Ala Asp Asp Phe Gln Thr Ser Ala Ser Pro Ala  
50 55 60

tcg gag gac aag atg act cct ttc gtt gtt gat acc aat gtt ttt gaa 240  
Ser Glu Asp Lys Met Thr Pro Phe Val Val Asp Thr Asn Val Phe Glu  
65 70 75 80

tcc gtc ttc aag aac acc gaa gat acc ctt cta gga gat atc gac aat 288  
Ser Val Phe Lys Asn Thr Glu Asp Thr Leu Leu Gly Asp Ile Asp Asn  
85 90 95

ggt ggt att gtt gac acg gag ttg aag gag atg ttc gat ttg gtt gac 336  
Val Gly Ile Val Asp Thr Glu Leu Lys Glu Met Phe Asp Leu Val Asp  
100 105 110

tcg gaa atc aat aac ggc act cct atc aag cag gaa gaa aag gat gat 384  
Ser Glu Ile Asn Asn Gly Thr Pro Ile Lys Gln Glu Glu Lys Asp Asp  
115 120 125

ttg gaa ttt act tca aga tcc cag tcc acc tca gct ctc ttg tcg tcg 432  
Leu Glu Phe Thr Ser Arg Ser Gln Ser Thr Ser Ala Leu Leu Ser Ser  
130 135 140

aaa tcg act tct gct tct cca gct gat gct gcc gct gca tgt gca agt 480  
Lys Ser Thr Ser Ala Ser Pro Ala Asp Ala Ala Ala Ala Cys Ala Ser  
145 150 155 160

cct tcg tca tcg tct tgt aaa aga tcc tat tct tct gct cag cta gaa 528  
Pro Ser Ser Ser Ser Cys Lys Arg Ser Tyr Ser Ser Ala Gln Leu Glu  
165 170 175

act acg ggt tcg gat gct cca aag aaa gat aag ctg ggc tgc acc cct 576  
Thr Thr Gly Ser Asp Ala Pro Lys Lys Asp Lys Leu Gly Cys Thr Pro  
180 185 190

tac act aga aaa cag aga aac aat cca tta cct ccg gtc att cca aag 624  
Tyr Thr Arg Lys Gln Arg Asn Asn Pro Leu Pro Pro Val Ile Pro Lys  
195 200 205

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ggt cag gat gtt gct tct atg aaa agg gca aga aac act gag gcc gca 672  
 Gly Gln Asp Val Ala Ser Met Lys Arg Ala Arg Asn Thr Glu Ala Ala  
 210 215 220

aga aga tca aga gcc aga aaa atg gaa aga atg tcc caa ctt gaa gaa 720  
 Arg Arg Ser Arg Ala Arg Lys Met Glu Arg Met Ser Gln Leu Glu Glu  
 225 230 235 240

aag tgt caa agc ttg ttg aag gaa aac gac gac ttg aaa gct caa gtt 768  
 Lys Cys Gln Ser Leu Leu Lys Glu Asn Asp Asp Leu Lys Ala Gln Val  
 245 250 255

caa gct ttg aag aaa tta ctt gga caa caa 798  
 Gln Ala Leu Lys Lys Leu Leu Gly Gln Gln  
 260 265

<210> SEQ ID NO 8  
 <211> LENGTH: 266  
 <212> TYPE: PRT  
 <213> ORGANISM: Drosophila sp.

<400> SEQUENCE: 8

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Ala Thr Ala Thr Thr Asn Thr Gly Glu Phe Leu Met Asn Glu Ser Pro  
 20 25 30

Arg Gln Glu Ala Gly Asp Leu Met Leu Asp Ser Leu Asp Phe Asn Ile  
 35 40 45

Met Gly Glu Asn Leu Ala Asp Asp Phe Gln Thr Ser Ala Ser Pro Ala  
 50 55 60

Ser Glu Asp Lys Met Thr Pro Phe Val Val Asp Thr Asn Val Phe Glu  
 65 70 75 80

Ser Val Phe Lys Asn Thr Glu Asp Thr Leu Leu Gly Asp Ile Asp Asn  
 85 90 95

Val Gly Ile Val Asp Thr Glu Leu Lys Glu Met Phe Asp Leu Val Asp  
 100 105 110

Ser Glu Ile Asn Asn Gly Thr Pro Ile Lys Gln Glu Glu Lys Asp Asp  
 115 120 125

Leu Glu Phe Thr Ser Arg Ser Gln Ser Thr Ser Ala Leu Leu Ser Ser  
 130 135 140

Lys Ser Thr Ser Ala Ser Pro Ala Asp Ala Ala Ala Cys Ala Ser  
 145 150 155 160

Pro Ser Ser Ser Ser Cys Lys Arg Ser Tyr Ser Ser Ala Gln Leu Glu  
 165 170 175

Thr Thr Gly Ser Asp Ala Pro Lys Lys Asp Lys Leu Gly Cys Thr Pro  
 180 185 190

Tyr Thr Arg Lys Gln Arg Asn Asn Pro Leu Pro Pro Val Ile Pro Lys  
 195 200 205

Gly Gln Asp Val Ala Ser Met Lys Arg Ala Arg Asn Thr Glu Ala Ala  
 210 215 220

Arg Arg Ser Arg Ala Arg Lys Met Glu Arg Met Ser Gln Leu Glu Glu  
 225 230 235 240

Lys Cys Gln Ser Leu Leu Lys Glu Asn Asp Asp Leu Lys Ala Gln Val  
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Gln Ala Leu Lys Lys Leu Leu Gly Gln Gln  
 260 265

<210> SEQ ID NO 9  
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&lt;212&gt; TYPE: PRT

&lt;213&gt; ORGANISM: Drosophila sp.

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 Gln Gln Leu Gln Gln Gln Gln Gln Gln Leu Gln Gln Gln Lys Ala Gln  
 35 40 45  
 Thr Gln Gln Gln Asn Ser Arg Lys Ile Lys Thr Gln Ala Thr Pro Thr  
 50 55 60  
 Leu Asn Gly Asn Gly Leu Leu Ser Gly Asn Pro Asn Gly Gly Gly Gly  
 65 70 75 80  
 Asp Ser Ser Pro Ser His Glu Val Asp His Pro Gly Gly Ala Gln Gly  
 85 90 95  
 Ala Gln Ala Ala Gly Gly Leu Pro Ser Leu Ser Gly Thr Pro Leu Arg  
 100 105 110  
 His His Lys Arg Ala Ser Ile Ser Thr Ala Ser Pro Pro Ile Arg Glu  
 115 120 125  
 Arg Arg Gly Thr Asn Thr Ser Ile Val Val Glu Leu Asp Gly Ser Gly  
 130 135 140  
 Ser Gly Ser Gly Ser Gly Gly Gly Gly Val Gly Val Gly Gln Gly Ala  
 145 150 155 160  
 Gly Cys Pro Pro Ser Gly Ser Cys Thr Ala Ser Gly Lys Ser Ser Arg  
 165 170 175  
 Glu Leu Ser Pro Ser Pro Lys Asn Gln Gln Gln Pro Arg Lys Met Ser  
 180 185 190  
 Gln Asp Tyr Arg Ser Arg Ala Gly Ser Phe Met His Leu Asp Asp Glu  
 195 200 205  
 Gly Arg Ser Leu Leu Met Arg Lys Pro Met Arg Leu Lys Asn Ile Glu  
 210 215 220  
 Gly Arg Pro Glu Val Tyr Asp Thr Leu His Cys Lys Gly Arg Glu Ile  
 225 230 235 240  
 Leu Ser Cys Ser Lys Ala Thr Cys Thr Ser Ser Ile Met Asn Ile Gly  
 245 250 255  
 Asn Ala Ala Val Glu Ala Arg Lys Ser Asp Leu Ile Leu Glu His Ala  
 260 265 270  
 Lys Asp Phe Leu Glu Gln Tyr Phe Thr Ser Ile Lys Arg Thr Ser Cys  
 275 280 285  
 Thr Ala His Glu Thr Arg Trp Lys Gln Val Arg Gln Ser Ile Glu Thr  
 290 295 300  
 Thr Gly His Tyr Gln Leu Thr Glu Thr Glu Leu Ile Tyr Gly Ala Lys  
 305 310 315 320  
 Leu Ala Trp Arg Asn Ser Ser Arg Cys Ile Gly Arg Ile Gln Trp Ser  
 325 330 335  
 Lys Leu Gln Val Phe Asp Cys Arg Tyr Val Thr Thr Thr Ser Gly Met  
 340 345 350  
 Phe Glu Ala Ile Cys Asn His Ile Lys Tyr Ala Thr Asn Lys Gly Asn  
 355 360 365  
 Leu Arg Ser Ala Ile Thr Ile Phe Pro Gln Arg Thr Asp Ala Lys His  
 370 375 380  
 Asp Tyr Arg Ile Trp Asn Asn Gln Leu Ile Ser Tyr Ala Gly Tyr Lys  
 385 390 395 400



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Gln Ala Asp Gly Lys Ile Ile Gly Asp Pro Met Asn Val Glu Phe Thr  
 405 410 415  
 Glu Val Cys Thr Lys Leu Gly Trp Lys Ser Lys Gly Ser Glu Trp Asp  
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 435 440 445  
 Asp Tyr Pro Pro Glu Leu Ile Leu Glu Val Pro Leu Thr His Pro Lys  
 450 455 460  
 Phe Glu Trp Phe Ser Asp Leu Gly Leu Arg Trp Tyr Ala Leu Pro Ala  
 465 470 475 480  
 Val Ser Ser Met Leu Phe Asp Val Gly Gly Ile Gln Phe Thr Ala Thr  
 485 490 495  
 Thr Phe Ser Gly Trp Tyr Met Ser Thr Glu Ile Gly Ser Arg Asn Leu  
 500 505 510  
 Cys Asp Thr Asn Arg Arg Asn Met Leu Glu Thr Val Ala Leu Lys Met  
 515 520 525  
 Gln Leu Asp Thr Arg Thr Pro Thr Ser Leu Trp Lys Asp Lys Ala Val  
 530 535 540  
 Val Glu Met Asn Ile Ala Val Leu His Ser Tyr Gln Ser Arg Asn Val  
 545 550 555 560  
 Thr Ile Val Asp His His Thr Ala Ser Glu Ser Phe Met Lys His Phe  
 565 570 575  
 Glu Asn Glu Ser Lys Leu Arg Asn Gly Cys Pro Ala Asp Trp Ile Trp  
 580 585 590  
 Ile Val Pro Pro Leu Ser Gly Ser Ile Thr Pro Val Phe His Gln Glu  
 595 600 605  
 Met Ala Leu Tyr Tyr Leu Lys Pro Ser Phe Glu Tyr Gln Asp Pro Ala  
 610 615 620  
 Trp Arg Thr His Val Trp Lys Lys Gly Arg Gly Glu Ser Lys Gly Lys  
 625 630 635 640  
 Lys Pro Arg Arg Lys Phe Asn Phe Lys Gln Ile Ala Arg Ala Val Lys  
 645 650 655  
 Phe Thr Ser Lys Leu Phe Gly Arg Ala Leu Ser Lys Arg Ile Lys Ala  
 660 665 670  
 Thr Val Leu Tyr Ala Thr Glu Thr Gly Lys Ser Glu Gln Tyr Ala Lys  
 675 680 685  
 Gln Leu Cys Glu Leu Leu Gly His Ala Phe Asn Ala Gln Ile Tyr Cys  
 690 695 700  
 Met Ser Asp Tyr Asp Ile Ser Ser Ile Glu His Glu Ala Leu Leu Ile  
 705 710 715 720  
 Val Val Ala Ser Thr Phe Gly Asn Gly Asp Pro Pro Glu Asn Gly Glu  
 725 730 735  
 Leu Phe Ser Gln Glu Leu Tyr Ala Met Arg Val Gln Glu Ser Ser Glu  
 740 745 750  
 His Gly Leu Gln Asp Ser Ser Ile Gly Ser Ser Lys Ser Phe Met Lys  
 755 760 765  
 Ala Ser Ser Arg Gln Glu Phe Met Lys Leu Pro Leu Gln Gln Val Lys  
 770 775 780  
 Arg Ile Asp Arg Trp Asp Ser Leu Arg Gly Ser Thr Ser Asp Thr Phe  
 785 790 795 800  
 Thr Glu Glu Thr Phe Gly Pro Leu Ser Asn Val Arg Phe Ala Val Phe  
 805 810 815

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Ala Leu Gly Ser Ser Ala Tyr Pro Asn Phe Cys Ala Phe Gly Gln Tyr  
820 825 830

Val Asp Asn Ile Leu Gly Glu Leu Gly Gly Glu Arg Leu Leu Arg Val  
835 840 845

Ala Tyr Gly Asp Glu Met Cys Gly Gln Glu Gln Ser Phe Arg Lys Trp  
850 855 860

Ala Pro Glu Val Phe Lys Leu Ala Cys Glu Thr Phe Cys Leu Asp Pro  
865 870 875 880

Glu Glu Ser Leu Ser Asp Ala Ser Leu Ala Leu Gln Asn Asp Ser Leu  
885 890 895

Thr Val Asn Thr Val Arg Leu Val Pro Ser Ala Asn Lys Gly Ser Leu  
900 905 910

Asp Ser Ser Leu Ser Lys Tyr His Asn Lys Lys Val His Cys Cys Lys  
915 920 925

Ala Lys Ala Lys Pro His Asn Leu Thr Arg Leu Ser Glu Gly Ala Lys  
930 935 940

Thr Thr Met Leu Leu Glu Ile Cys Ala Pro Gly Leu Glu Tyr Glu Pro  
945 950 955 960

Gly Asp His Val Gly Ile Phe Pro Ala Asn Arg Thr Glu Leu Val Asp  
965 970 975

Gly Leu Leu Asn Arg Leu Val Gly Val Asp Asn Pro Asp Glu Val Leu  
980 985 990

Gln Leu Gln Leu Leu Lys Glu Lys Gln Thr Ser Asn Gly Ile Phe Lys  
995 1000 1005

Cys Trp Glu Pro His Asp Lys Ile Pro Pro Asp Thr Leu Arg Asn  
1010 1015 1020

Leu Leu Ala Arg Phe Phe Asp Leu Thr Thr Pro Pro Ser Arg Gln  
1025 1030 1035

Leu Leu Thr Leu Leu Ala Gly Phe Cys Glu Asp Thr Ala Asp Lys  
1040 1045 1050

Glu Arg Leu Glu Leu Leu Val Asn Asp Ser Ser Ala Tyr Glu Asp  
1055 1060 1065

Trp Arg His Trp Arg Leu Pro His Leu Leu Asp Val Leu Glu Glu  
1070 1075 1080

Phe Pro Ser Cys Arg Pro Pro Ala Pro Leu Leu Leu Ala Gln Leu  
1085 1090 1095

Thr Pro Leu Gln Pro Arg Phe Tyr Ser Ile Ser Ser Ser Pro Arg  
1100 1105 1110

Arg Val Ser Asp Glu Ile His Leu Thr Val Ala Ile Val Lys Tyr  
1115 1120 1125

Arg Cys Glu Asp Gly Gln Gly Asp Glu Arg Tyr Gly Val Cys Ser  
1130 1135 1140

Asn Tyr Leu Ser Gly Leu Arg Ala Asp Asp Glu Leu Phe Met Phe  
1145 1150 1155

Val Arg Ser Ala Leu Gly Phe His Leu Pro Ser Asp Arg Ser Arg  
1160 1165 1170

Pro Ile Ile Leu Ile Gly Pro Gly Thr Gly Ile Ala Pro Phe Arg  
1175 1180 1185

Ser Phe Trp Gln Glu Phe Gln Val Leu Arg Asp Leu Asp Pro Thr  
1190 1195 1200

Ala Lys Leu Pro Lys Met Trp Leu Phe Phe Gly Cys Arg Asn Arg  
1205 1210 1215

Asp Val Asp Leu Tyr Ala Glu Glu Lys Ala Glu Leu Gln Lys Asp

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1220	1225	1230
Gln Ile Leu Asp Arg Val Phe Leu Ala Leu Ser Arg Glu Gln Ala 1235	1240	1245
Ile Pro Lys Thr Tyr Val Gln Asp Leu Ile Glu Gln Glu Phe Asp 1250	1255	1260
Ser Leu Tyr Gln Leu Ile Val Gln Glu Arg Gly His Ile Tyr Val 1265	1270	1275
Cys Gly Asp Val Thr Met Ala Glu His Val Tyr Gln Thr Ile Arg 1280	1285	1290
Lys Cys Ile Ala Gly Lys Glu Gln Lys Ser Glu Ala Glu Val Glu 1295	1300	1305
Thr Phe Leu Leu Thr Leu Arg Asp Glu Ser Arg Tyr His Glu Asp 1310	1315	1320
Ile Phe Gly Ile Thr Leu Arg Thr Ala Glu Ile His Thr Lys Ser 1325	1330	1335
Arg Ala Thr Ala Arg Ile Arg Met Ala Ser Gln Pro 1340	1345	1350
 <210> SEQ ID NO 10 <211> LENGTH: 1205 <212> TYPE: PRT <213> ORGANISM: Bos sp.		
 <400> SEQUENCE: 10		
Met Gly Asn Leu Lys Ser Val Gly Gln Glu Pro Gly Pro Pro Cys Gly 1	5	10
Leu Gly Leu Gly Leu Gly Leu Gly Leu Cys Gly Lys Gln Gly Pro Ala 20	25	30
Ser Pro Ala Pro Glu Pro Ser Arg Ala Pro Ala Pro Ala Thr Pro His 35	40	45
Ala Pro Asp His Ser Pro Ala Pro Asn Ser Pro Thr Leu Thr Arg Pro 50	55	60
Pro Glu Gly Pro Lys Phe Pro Arg Val Lys Asn Trp Glu Leu Gly Ser 65	70	75
Ile Thr Tyr Asp Thr Leu Cys Ala Gln Ser Gln Gln Asp Gly Pro Cys 85	90	95
Thr Pro Arg Arg Cys Leu Gly Ser Leu Val Leu Pro Arg Lys Leu Gln 100	105	110
Thr Arg Pro Ser Pro Gly Pro Pro Pro Ala Glu Gln Leu Leu Ser Gln 115	120	125
Ala Arg Asp Phe Ile Asn Gln Tyr Tyr Ser Ser Ile Lys Arg Ser Gly 130	135	140
Ser Gln Ala His Glu Glu Arg Leu Gln Glu Val Glu Ala Glu Val Ala 145	150	155
Ser Thr Gly Thr Ile His Leu Arg Glu Ser Glu Leu Val Phe Gly Ala 165	170	175
Lys Gln Ala Trp Arg Asn Ala Pro Arg Cys Val Gly Arg Ile Gln Trp 180	185	190
Gly Lys Leu Gln Val Phe Asp Ala Arg Asp Cys Ser Ser Ala Gln Glu 195	200	205
Met Phe Thr Tyr Ile Cys Asn His Ile Lys Tyr Ala Thr Asn Arg Gly 210	215	220
Asn Leu Arg Ser Ala Ile Thr Val Phe Pro Gln Arg Ala Pro Gly Arg 225	230	235
		240

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Gly	Asp	Phe	Arg	Ile	Trp	Asn	Ser	Gln	Leu	Val	Arg	Tyr	Ala	Gly	Tyr
				245					250					255	
Arg	Gln	Gln	Asp	Gly	Ser	Val	Arg	Gly	Asp	Pro	Ala	Asn	Val	Glu	Ile
			260					265					270		
Thr	Glu	Leu	Cys	Ile	Gln	His	Gly	Trp	Thr	Pro	Gly	Asn	Gly	Arg	Phe
		275					280					285			
Asp	Val	Leu	Pro	Leu	Leu	Leu	Gln	Ala	Pro	Asp	Glu	Ala	Pro	Glu	Leu
	290					295					300				
Phe	Val	Leu	Pro	Pro	Glu	Leu	Val	Leu	Glu	Val	Pro	Leu	Gly	Ala	Pro
305					310					315					320
His	Thr	Gly	Val	Val	Arg	Gly	Pro	Gly	Leu	Arg	Trp	Tyr	Ala	Leu	Pro
				325					330					335	
Ala	Val	Ser	Asn	Met	Leu	Leu	Glu	Ile	Gly	Gly	Leu	Glu	Phe	Ser	Ala
			340					345					350		
Ala	Pro	Phe	Ser	Gly	Trp	Tyr	Met	Ser	Thr	Glu	Ile	Gly	Thr	Arg	Asn
		355					360					365			
Leu	Cys	Asp	Pro	His	Arg	Tyr	Asn	Ile	Leu	Glu	Asp	Val	Ala	Val	Cys
	370					375					380				
Met	Asp	Leu	Asp	Thr	Arg	Thr	Thr	Ser	Ser	Leu	Trp	Lys	Asp	Lys	Ala
385					390					395					400
Ala	Val	Glu	Ile	Asn	Leu	Ala	Val	Leu	His	Ser	Phe	Gln	Leu	Ala	Lys
				405					410					415	
Val	Thr	Ile	Val	Asp	His	His	Ala	Ala	Thr	Val	Ser	Phe	Met	Lys	His
			420					425					430		
Leu	Asp	Asn	Glu	Gln	Lys	Ala	Arg	Gly	Gly	Cys	Pro	Ala	Asp	Trp	Ala
		435					440					445			
Trp	Ile	Val	Pro	Pro	Ile	Tyr	Gly	Ser	Leu	Pro	Pro	Val	Phe	His	Gln
	450					455					460				
Glu	Met	Val	Asn	Tyr	Ile	Leu	Ser	Pro	Ala	Phe	Arg	Tyr	Gln	Pro	Asp
465					470					475					480
Pro	Trp	Lys	Gly	Ser	Ala	Thr	Lys	Gly	Ala	Gly	Ile	Thr	Arg	Lys	Lys
				485					490					495	
Thr	Phe	Lys	Glu	Val	Ala	Asn	Ala	Val	Lys	Ile	Ser	Ala	Ser	Leu	Met
			500					505					510		
Gly	Thr	Leu	Met	Ala	Lys	Arg	Val	Lys	Ala	Thr	Ile	Leu	Tyr	Ala	Ser
		515					520					525			
Glu	Thr	Gly	Arg	Ala	Gln	Ser	Tyr	Ala	Gln	Gln	Leu	Gly	Arg	Leu	Phe
	530					535					540				
Arg	Lys	Ala	Phe	Asp	Pro	Arg	Val	Leu	Cys	Met	Asp	Glu	Tyr	Asp	Val
545					550					555					560
Val	Ser	Leu	Glu	His	Glu	Ala	Leu	Val	Leu	Val	Val	Thr	Ser	Thr	Phe
				565					570					575	
Gly	Asn	Gly	Asp	Pro	Pro	Glu	Asn	Gly	Glu	Ser	Phe	Ala	Ala	Ala	Leu
			580					585					590		
Met	Glu	Met	Ser	Gly	Pro	Tyr	Asn	Ser	Ser	Pro	Arg	Pro	Glu	Gln	His
	595						600					605			
Lys	Ser	Tyr	Lys	Ile	Arg	Phe	Asn	Ser	Val	Ser	Cys	Ser	Asp	Pro	Leu
	610					615					620				
Val	Ser	Ser	Trp	Arg	Arg	Lys	Arg	Lys	Glu	Ser	Ser	Asn	Thr	Asp	Ser
625					630					635					640
Ala	Gly	Ala	Leu	Gly	Thr	Leu	Arg	Phe	Cys	Val	Phe	Gly	Leu	Gly	Ser
				645					650					655	
Arg	Ala	Tyr	Pro	His	Phe	Cys	Ala	Phe	Ala	Arg	Ala	Val	Asp	Thr	Arg

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660					665					670					
Leu	Glu	Glu	Leu	Gly	Gly	Glu	Arg	Leu	Leu	Gln	Leu	Gly	Gln	Gly	Asp
	675						680					685			
Glu	Leu	Cys	Gly	Gln	Glu	Glu	Ala	Phe	Arg	Gly	Trp	Ala	Lys	Ala	Ala
	690						695					700			
Phe	Gln	Ala	Ser	Cys	Glu	Thr	Phe	Cys	Val	Gly	Glu	Glu	Ala	Lys	Ala
	705						710					715			720
Ala	Ala	Gln	Asp	Ile	Phe	Ser	Pro	Lys	Arg	Ser	Trp	Lys	Arg	Gln	Arg
				725					730					735	
Tyr	Arg	Leu	Ser	Ala	Gln	Ala	Glu	Gly	Leu	Gln	Leu	Leu	Pro	Gly	Leu
			740						745					750	
Ile	His	Val	His	Arg	Arg	Lys	Met	Phe	Gln	Ala	Thr	Val	Leu	Ser	Val
		755					760						765		
Glu	Asn	Leu	Gln	Ser	Ser	Lys	Ser	Thr	Arg	Ala	Thr	Ile	Leu	Val	Arg
		770					775					780			
Leu	Asp	Thr	Ala	Gly	Gln	Glu	Gly	Leu	Gln	Tyr	Gln	Pro	Gly	Asp	His
	785						790					795			800
Ile	Gly	Ile	Ser	Ala	Pro	Asn	Arg	Pro	Gly	Leu	Val	Glu	Ala	Leu	Leu
				805					810					815	
Ser	Arg	Val	Glu	Asp	Pro	Pro	Pro	Pro	Thr	Glu	Ser	Val	Ala	Val	Glu
			820						825				830		
Gln	Leu	Glu	Lys	Gly	Ser	Pro	Gly	Gly	Pro	Pro	Pro	Ser	Trp	Val	Arg
		835					840					845			
Asp	Pro	Arg	Leu	Pro	Pro	Cys	Thr	Val	Arg	Gln	Ala	Leu	Thr	Phe	Phe
	850						855					860			
Leu	Asp	Ile	Thr	Ser	Pro	Pro	Ser	Pro	Arg	Leu	Leu	Arg	Leu	Leu	Ser
	865						870					875			880
Thr	Leu	Ala	Glu	Glu	Pro	Ser	Glu	Gln	Gln	Glu	Leu	Glu	Thr	Leu	Ser
				885					890					895	
Gln	Asp	Pro	Arg	Arg	Tyr	Glu	Glu	Trp	Lys	Leu	Val	Arg	Cys	Pro	Thr
			900					905					910		
Leu	Leu	Glu	Val	Leu	Glu	Gln	Phe	Pro	Ser	Val	Ala	Leu	Pro	Ala	Pro
		915					920					925			
Leu	Leu	Leu	Thr	Gln	Leu	Pro	Leu	Leu	Gln	Pro	Arg	Tyr	Tyr	Ser	Val
		930					935					940			
Ser	Ser	Ala	Pro	Asn	Ala	His	Pro	Gly	Glu	Val	His	Leu	Thr	Val	Ala
				945			950					955			960
Val	Leu	Ala	Tyr	Arg	Thr	Gln	Asp	Gly	Leu	Gly	Pro	Leu	His	Tyr	Gly
				965					970					975	
Val	Cys	Ser	Thr	Trp	Leu	Ser	Gln	Leu	Lys	Thr	Gly	Asp	Pro	Val	Pro
			980						985				990		
Cys	Phe	Ile	Arg	Gly	Ala	Pro	Ser	Phe	Arg	Leu	Pro	Pro	Asp	Pro	Tyr
		995					1000					1005			
Val	Pro	Cys	Ile	Leu	Val	Gly	Pro	Gly	Thr	Gly	Ile	Ala	Pro	Phe	
		1010					1015					1020			
Arg	Gly	Phe	Trp	Gln	Glu	Arg	Leu	His	Asp	Ile	Glu	Ser	Lys	Gly	
		1025					1030					1035			
Leu	Gln	Pro	His	Pro	Met	Thr	Leu	Val	Phe	Gly	Cys	Arg	Cys	Ser	
		1040					1045					1050			
Gln	Leu	Asp	His	Leu	Tyr	Arg	Asp	Glu	Val	Gln	Asp	Ala	Gln	Glu	
		1055					1060					1065			
Arg	Gly	Val	Phe	Gly	Arg	Val	Leu	Thr	Ala	Phe	Ser	Arg	Glu	Pro	
		1070					1075					1080			

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Asp Ser Pro Lys Thr Tyr Val Gln Asp Ile Leu Arg Thr Glu Leu  
 1085 1090 1095  
 Ala Ala Glu Val His Arg Val Leu Cys Leu Glu Arg Gly His Met  
 1100 1105 1110  
 Phe Val Cys Gly Asp Val Thr Met Ala Thr Ser Val Leu Gln Thr  
 1115 1120 1125  
 Val Gln Arg Ile Leu Ala Thr Glu Gly Asp Met Glu Leu Asp Glu  
 1130 1135 1140  
 Ala Gly Asp Val Ile Gly Val Leu Arg Asp Gln Gln Arg Tyr His  
 1145 1150 1155  
 Glu Asp Ile Phe Gly Leu Thr Leu Arg Thr Gln Glu Val Thr Ser  
 1160 1165 1170  
 Arg Ile Arg Thr Gln Ser Phe Ser Leu Gln Glu Arg His Leu Arg  
 1175 1180 1185  
 Gly Ala Val Pro Trp Ala Phe Asp Pro Pro Gly Pro Asp Thr Pro  
 1190 1195 1200  
 Gly Pro  
 1205

<210> SEQ ID NO 11  
 <211> LENGTH: 1429  
 <212> TYPE: PRT  
 <213> ORGANISM: Rattus sp.

<400> SEQUENCE: 11

Met Glu Glu Asn Thr Phe Gly Val Gln Gln Ile Gln Pro Asn Val Ile  
 1 5 10 15  
 Ser Val Arg Leu Phe Lys Arg Lys Val Gly Gly Leu Gly Phe Leu Val  
 20 25 30  
 Lys Glu Arg Val Ser Lys Pro Pro Val Ile Ile Ser Asp Leu Ile Arg  
 35 40 45  
 Gly Gly Ala Ala Glu Gln Ser Gly Leu Ile Gln Ala Gly Asp Ile Ile  
 50 55 60  
 Leu Ala Val Asn Asp Arg Pro Leu Val Asp Leu Ser Tyr Asp Ser Ala  
 65 70 75 80  
 Leu Glu Val Leu Arg Gly Ile Ala Ser Glu Thr His Val Val Leu Ile  
 85 90 95  
 Leu Arg Gly Pro Glu Gly Phe Thr Thr His Leu Glu Thr Thr Phe Thr  
 100 105 110  
 Gly Asp Gly Thr Pro Lys Thr Ile Arg Val Thr Gln Pro Leu Gly Pro  
 115 120 125  
 Pro Thr Lys Ala Val Asp Leu Ser His Gln Pro Ser Ala Ser Lys Asp  
 130 135 140  
 Gln Ser Leu Ala Val Asp Arg Val Thr Gly Leu Gly Asn Gly Pro Gln  
 145 150 155 160  
 His Ala Gln Gly His Gly Gln Gly Ala Gly Ser Val Ser Gln Ala Asn  
 165 170 175  
 Gly Val Ala Ile Asp Pro Thr Met Lys Ser Thr Lys Ala Asn Leu Gln  
 180 185 190  
 Asp Ile Gly Glu His Asp Glu Leu Leu Lys Glu Ile Glu Pro Val Leu  
 195 200 205  
 Ser Ile Leu Asn Ser Gly Ser Lys Ala Thr Asn Arg Gly Gly Pro Ala  
 210 215 220  
 Lys Ala Glu Met Lys Asp Thr Gly Ile Gln Val Asp Arg Asp Leu Asp

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225					230										235				240
Gly	Lys	Ser	His	Lys	Ala	Pro	Pro	Leu	Gly	Gly	Asp	Asn	Asp	Arg	Val				
				245					250					255					
Phe	Asn	Asp	Leu	Trp	Gly	Lys	Asp	Asn	Val	Pro	Val	Ile	Leu	Asn	Asn				
			260					265					270						
Pro	Tyr	Ser	Glu	Lys	Glu	Gln	Ser	Pro	Thr	Ser	Gly	Lys	Gln	Ser	Pro				
		275					280					285							
Thr	Lys	Asn	Gly	Ser	Pro	Ser	Arg	Cys	Pro	Arg	Phe	Leu	Lys	Val	Lys				
	290					295					300								
Asn	Trp	Glu	Thr	Asp	Val	Val	Leu	Thr	Asp	Thr	Leu	His	Leu	Lys	Ser				
305					310					315					320				
Thr	Leu	Glu	Thr	Gly	Cys	Thr	Glu	His	Ile	Cys	Met	Gly	Ser	Ile	Met				
				325					330					335					
Leu	Pro	Ser	Gln	His	Thr	Arg	Lys	Pro	Glu	Asp	Val	Arg	Thr	Lys	Asp				
			340					345					350						
Gln	Leu	Phe	Pro	Leu	Ala	Lys	Glu	Phe	Leu	Asp	Gln	Tyr	Tyr	Ser	Ser				
		355					360					365							
Ile	Lys	Arg	Phe	Gly	Ser	Lys	Ala	His	Met	Asp	Arg	Leu	Glu	Glu	Val				
	370					375					380								
Asn	Lys	Glu	Ile	Glu	Ser	Thr	Ser	Thr	Tyr	Gln	Leu	Lys	Asp	Thr	Glu				
385					390					395				400					
Leu	Ile	Tyr	Gly	Ala	Lys	His	Ala	Trp	Arg	Asn	Ala	Ser	Arg	Cys	Val				
			405					410					415						
Gly	Arg	Ile	Gln	Trp	Ser	Lys	Leu	Gln	Val	Phe	Asp	Ala	Arg	Asp	Cys				
			420					425					430						
Thr	Thr	Ala	His	Gly	Met	Phe	Asn	Tyr	Ile	Cys	Asn	His	Val	Lys	Tyr				
		435					440					445							
Ala	Thr	Asn	Lys	Gly	Asn	Leu	Arg	Ser	Ala	Ile	Thr	Ile	Phe	Pro	Gln				
	450					455					460								
Arg	Thr	Asp	Gly	Lys	His	Asp	Phe	Arg	Val	Trp	Asn	Ser	Gln	Leu	Ile				
465					470					475				480					
Arg	Tyr	Ala	Gly	Tyr	Lys	Gln	Pro	Asp	Gly	Ser	Thr	Leu	Gly	Asp	Pro				
			485						490					495					
Ala	Asn	Val	Gln	Phe	Thr	Glu	Ile	Cys	Ile	Gln	Gln	Gly	Trp	Lys	Ala				
		500						505					510						
Pro	Arg	Gly	Arg	Phe	Asp	Val	Leu	Pro	Leu	Leu	Leu	Gln	Ala	Asn	Gly				
		515					520					525							
Asn	Asp	Pro	Glu	Leu	Phe	Gln	Ile	Pro	Pro	Glu	Leu	Val	Leu	Glu	Val				
	530					535						540							
Pro	Ile	Arg	His	Pro	Lys	Phe	Asp	Trp	Phe	Lys	Asp	Leu	Gly	Leu	Lys				
545					550					555				560					
Trp	Tyr	Gly	Leu	Pro	Ala	Val	Ser	Asn	Met	Leu	Leu	Glu	Ile	Gly	Gly				
				565					570					575					
Leu	Glu	Phe	Ser	Ala	Cys	Pro	Phe	Ser	Gly	Trp	Tyr	Met	Gly	Thr	Glu				
			580					585					590						
Ile	Gly	Val	Arg	Asp	Tyr	Cys	Asp	Asn	Ser	Arg	Tyr	Asn	Ile	Leu	Glu				
	595						600					605							
Glu	Val	Ala	Lys	Lys	Met	Asp	Leu	Asp	Met	Arg	Lys	Thr	Ser	Ser	Leu				
	610					615					620								
Trp	Lys	Asp	Gln	Ala	Leu	Val	Glu	Ile	Asn	Ile	Ala	Val	Leu	Tyr	Ser				
625					630					635				640					
Phe	Gln	Ser	Asp	Lys	Val	Thr	Ile	Val	Asp	His	His	Ser	Ala	Thr	Glu				
				645					650					655					

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Ser Phe Ile Lys His Met Glu Asn Glu Tyr Arg Cys Arg Gly Gly Cys  
                   660                  665                  670

Pro Ala Asp Trp Val Trp Ile Val Pro Pro Met Ser Gly Ser Ile Thr  
                   675                  680                  685

Pro Val Phe His Gln Glu Met Leu Asn Tyr Arg Leu Thr Pro Ser Phe  
                   690                  695                  700

Glu Tyr Gln Pro Asp Pro Trp Asn Thr His Val Trp Lys Gly Thr Asn  
 705                  710                  715                  720

Gly Thr Pro Thr Lys Arg Arg Ala Ile Gly Phe Lys Lys Leu Ala Glu  
                   725                  730                  735

Ala Val Lys Phe Ser Ala Lys Leu Met Gly Gln Ala Met Ala Lys Arg  
                   740                  745                  750

Val Lys Ala Thr Ile Leu Tyr Ala Thr Glu Thr Gly Lys Ser Gln Ala  
                   755                  760                  765

Tyr Ala Lys Thr Leu Cys Glu Ile Phe Lys His Ala Phe Asp Ala Lys  
                   770                  775                  780

Ala Met Ser Met Glu Glu Tyr Asp Ile Val His Leu Glu His Glu Ala  
 785                  790                  795                  800

Leu Val Leu Val Val Thr Ser Thr Phe Gly Asn Gly Asp Pro Pro Glu  
                   805                  810                  815

Asn Gly Glu Lys Phe Gly Cys Ala Leu Met Glu Met Arg His Pro Asn  
                   820                  825                  830

Ser Val Gln Glu Glu Arg Lys Ser Tyr Lys Val Arg Phe Asn Ser Val  
                   835                  840                  845

Ser Ser Tyr Ser Asp Ser Arg Lys Ser Ser Gly Asp Gly Pro Asp Leu  
                   850                  855                  860

Arg Asp Asn Phe Glu Ser Thr Gly Pro Leu Ala Asn Val Arg Phe Ser  
 865                  870                  875                  880

Val Phe Gly Leu Gly Ser Arg Ala Tyr Pro His Phe Cys Ala Phe Gly  
                   885                  890                  895

His Ala Val Asp Thr Leu Leu Glu Glu Leu Gly Gly Glu Arg Ile Leu  
                   900                  905                  910

Lys Met Arg Glu Gly Asp Glu Leu Cys Gly Gln Glu Glu Ala Phe Arg  
                   915                  920                  925

Thr Trp Ala Lys Lys Val Phe Lys Ala Ala Cys Asp Val Phe Cys Val  
                   930                  935                  940

Gly Asp Asp Val Asn Ile Glu Lys Pro Asn Asn Ser Leu Ile Ser Asn  
 945                  950                  955                  960

Asp Arg Ser Trp Lys Arg Asn Lys Phe Arg Leu Thr Tyr Val Ala Glu  
                   965                  970                  975

Ala Pro Asp Leu Thr Gln Gly Leu Ser Asn Val His Lys Lys Arg Val  
                   980                  985                  990

Ser Ala Ala Arg Leu Leu Ser Arg Gln Asn Leu Gln Ser Pro Lys Phe  
                   995                  1000                  1005

Ser Arg Ser Thr Ile Phe Val Arg Leu His Thr Asn Gly Asn Gln  
                   1010                  1015                  1020

Glu Leu Gln Tyr Gln Pro Gly Asp His Leu Gly Val Phe Pro Gly  
                   1025                  1030                  1035

Asn His Glu Asp Leu Val Asn Ala Leu Ile Glu Arg Leu Glu Asp  
                   1040                  1045                  1050

Ala Pro Pro Ala Asn His Val Val Lys Val Glu Met Leu Glu Glu  
                   1055                  1060                  1065



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Arg	Asn	Thr	Ala	Leu	Gly	Val	Ile	Ser	Asn	Trp	Lys	Asp	Glu	Ser
1070						1075					1080			
Arg	Leu	Pro	Pro	Cys	Thr	Ile	Phe	Gln	Ala	Phe	Lys	Tyr	Tyr	Leu
1085						1090					1095			
Asp	Ile	Thr	Thr	Pro	Pro	Thr	Pro	Leu	Gln	Leu	Gln	Gln	Phe	Ala
1100						1105					1110			
Ser	Leu	Ala	Thr	Asn	Glu	Lys	Glu	Lys	Gln	Arg	Leu	Leu	Val	Leu
1115						1120					1125			
Ser	Lys	Gly	Leu	Gln	Glu	Tyr	Glu	Glu	Trp	Lys	Trp	Gly	Lys	Asn
1130						1135					1140			
Pro	Thr	Met	Val	Glu	Val	Leu	Glu	Glu	Phe	Pro	Ser	Ile	Gln	Met
1145						1150					1155			
Pro	Ala	Thr	Leu	Leu	Leu	Thr	Gln	Leu	Ser	Leu	Leu	Gln	Pro	Arg
1160						1165					1170			
Tyr	Tyr	Ser	Ile	Ser	Ser	Ser	Pro	Asp	Met	Tyr	Pro	Asp	Glu	Val
1175						1180					1185			
His	Leu	Thr	Val	Ala	Ile	Val	Ser	Tyr	His	Thr	Arg	Asp	Gly	Glu
1190						1195					1200			
Gly	Pro	Val	His	His	Gly	Val	Cys	Ser	Ser	Trp	Leu	Asn	Arg	Ile
1205						1210					1215			
Gln	Ala	Asp	Asp	Val	Val	Pro	Cys	Phe	Val	Arg	Gly	Ala	Pro	Ser
1220						1225					1230			
Phe	His	Leu	Pro	Arg	Asn	Pro	Gln	Val	Pro	Cys	Ile	Leu	Val	Gly
1235						1240					1245			
Pro	Gly	Thr	Gly	Ile	Ala	Pro	Phe	Arg	Ser	Phe	Trp	Gln	Gln	Arg
1250						1255					1260			
Gln	Phe	Asp	Ile	Gln	His	Lys	Gly	Met	Asn	Pro	Cys	Pro	Met	Val
1265						1270					1275			
Leu	Val	Phe	Gly	Cys	Arg	Gln	Ser	Lys	Ile	Asp	His	Ile	Tyr	Arg
1280						1285					1290			
Glu	Glu	Thr	Leu	Gln	Ala	Lys	Asn	Lys	Gly	Val	Phe	Arg	Glu	Leu
1295						1300					1305			
Tyr	Thr	Ala	Tyr	Ser	Arg	Glu	Pro	Asp	Arg	Pro	Lys	Lys	Tyr	Val
1310						1315					1320			
Gln	Asp	Val	Leu	Gln	Glu	Gln	Leu	Ala	Glu	Ser	Val	Tyr	Arg	Ala
1325						1330					1335			
Leu	Lys	Glu	Gln	Gly	Gly	His	Ile	Tyr	Val	Cys	Gly	Asp	Val	Thr
1340						1345					1350			
Met	Ala	Ala	Asp	Val	Leu	Lys	Ala	Ile	Gln	Arg	Ile	Met	Thr	Gln
1355						1360					1365			
Gln	Gly	Lys	Leu	Ser	Glu	Glu	Asp	Ala	Gly	Val	Phe	Ile	Ser	Arg
1370						1375					1380			
Leu	Arg	Asp	Asp	Asn	Arg	Tyr	His	Glu	Asp	Ile	Phe	Gly	Val	Thr
1385						1390					1395			
Leu	Arg	Thr	Tyr	Glu	Val	Thr	Asn	Arg	Leu	Arg	Ser	Glu	Ser	Ile
1400						1405					1410			
Ala	Phe	Ile	Glu	Glu	Ser	Lys	Lys	Asp	Ala	Asp	Glu	Val	Phe	Ser
1415						1420					1425			

Ser

<210> SEQ ID NO 12  
 <211> LENGTH: 1144  
 <212> TYPE: PRT  
 <213> ORGANISM: Mus sp.

-continued

&lt;400&gt; SEQUENCE: 12

Met Ala Cys Pro Trp Lys Phe Leu Phe Lys Val Lys Ser Tyr Gln Ser  
 1 5 10 15  
 Asp Leu Lys Glu Glu Lys Asp Ile Asn Asn Asn Val Lys Lys Thr Pro  
 20 25 30  
 Cys Ala Val Leu Ser Pro Thr Ile Gln Asp Asp Pro Lys Ser His Gln  
 35 40 45  
 Asn Gly Ser Pro Gln Leu Leu Thr Gly Thr Ala Gln Asn Val Pro Glu  
 50 55 60  
 Ser Leu Asp Lys Leu His Val Thr Ser Thr Arg Pro Gln Tyr Val Arg  
 65 70 75 80  
 Ile Lys Asn Trp Gly Ser Gly Glu Ile Leu His Asp Thr Leu His His  
 85 90 95  
 Lys Ala Thr Ser Asp Phe Thr Cys Lys Ser Lys Ser Cys Leu Gly Ser  
 100 105 110  
 Ile Met Asn Pro Lys Ser Leu Thr Arg Gly Pro Arg Asp Lys Pro Thr  
 115 120 125  
 Pro Leu Glu Glu Leu Leu Pro His Ala Ile Glu Phe Ile Asn Gln Tyr  
 130 135 140  
 Tyr Gly Ser Phe Lys Glu Ala Lys Ile Glu Glu His Leu Ala Arg Leu  
 145 150 155 160  
 Glu Ala Val Thr Lys Glu Ile Glu Thr Thr Gly Thr Tyr Gln Leu Thr  
 165 170 175  
 Leu Asp Glu Leu Ile Phe Ala Thr Lys Met Ala Trp Arg Asn Ala Pro  
 180 185 190  
 Arg Cys Ile Gly Arg Ile Gln Trp Ser Asn Leu Gln Val Phe Asp Ala  
 195 200 205  
 Arg Asn Cys Ser Thr Ala Gln Glu Met Phe Gln His Ile Cys Arg His  
 210 215 220  
 Ile Leu Tyr Ala Thr Asn Asn Gly Asn Ile Arg Ser Ala Ile Thr Val  
 225 230 235 240  
 Phe Pro Gln Arg Ser Asp Gly Lys His Asp Phe Arg Leu Trp Asn Ser  
 245 250 255  
 Gln Leu Ile Arg Tyr Ala Gly Tyr Gln Met Pro Asp Gly Thr Ile Arg  
 260 265 270  
 Gly Asp Ala Ala Thr Leu Glu Phe Thr Gln Leu Cys Ile Asp Leu Gly  
 275 280 285  
 Trp Lys Pro Arg Tyr Gly Arg Phe Asp Val Leu Pro Leu Val Leu Gln  
 290 295 300  
 Ala Asp Gly Gln Asp Pro Glu Val Phe Glu Ile Pro Pro Asp Leu Val  
 305 310 315 320  
 Leu Glu Val Thr Met Glu His Pro Lys Tyr Glu Trp Phe Gln Glu Leu  
 325 330 335  
 Gly Leu Lys Trp Tyr Ala Leu Pro Ala Val Ala Asn Met Leu Leu Glu  
 340 345 350  
 Val Gly Gly Leu Glu Phe Pro Ala Cys Pro Phe Asn Gly Trp Tyr Met  
 355 360 365  
 Gly Thr Glu Ile Gly Val Arg Asp Phe Cys Asp Thr Gln Arg Tyr Asn  
 370 375 380  
 Ile Leu Glu Glu Val Gly Arg Arg Met Gly Leu Glu Thr His Thr Leu  
 385 390 395 400  
 Ala Ser Leu Trp Lys Asp Arg Ala Val Thr Glu Ile Asn Val Ala Val

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405					410					415					
Leu	His	Ser	Phe	Gln	Lys	Gln	Asn	Val	Thr	Ile	Met	Asp	His	His	Thr
			420					425					430		
Ala	Ser	Glu	Ser	Phe	Met	Lys	His	Met	Gln	Asn	Glu	Tyr	Arg	Ala	Arg
		435					440					445			
Gly	Gly	Cys	Pro	Ala	Asp	Trp	Ile	Trp	Leu	Val	Pro	Pro	Val	Ser	Gly
	450					455					460				
Ser	Ile	Thr	Pro	Val	Phe	His	Gln	Glu	Met	Leu	Asn	Tyr	Val	Leu	Ser
465					470					475					480
Pro	Phe	Tyr	Tyr	Tyr	Gln	Ile	Glu	Pro	Trp	Lys	Thr	His	Ile	Trp	Gln
				485					490					495	
Asn	Glu	Lys	Leu	Arg	Pro	Arg	Arg	Arg	Glu	Ile	Arg	Phe	Arg	Val	Leu
			500					505					510		
Val	Lys	Val	Val	Phe	Phe	Ala	Ser	Met	Leu	Met	Arg	Lys	Val	Met	Ala
		515					520					525			
Ser	Arg	Val	Arg	Ala	Thr	Val	Leu	Phe	Ala	Thr	Glu	Thr	Gly	Lys	Ser
	530					535					540				
Glu	Ala	Leu	Ala	Arg	Asp	Leu	Ala	Thr	Leu	Phe	Ser	Tyr	Ala	Phe	Asn
545					550					555					560
Thr	Lys	Val	Val	Cys	Met	Asp	Gln	Tyr	Lys	Ala	Ser	Thr	Leu	Glu	Glu
				565					570					575	
Glu	Gln	Leu	Leu	Leu	Val	Val	Thr	Ser	Thr	Phe	Gly	Asn	Gly	Asp	Cys
		580						585					590		
Pro	Ser	Asn	Gly	Gln	Thr	Leu	Lys	Lys	Ser	Leu	Phe	Met	Leu	Arg	Glu
		595					600					605			
Leu	Asn	His	Thr	Phe	Arg	Tyr	Ala	Val	Phe	Gly	Leu	Gly	Ser	Ser	Met
	610					615					620				
Tyr	Pro	Gln	Phe	Cys	Ala	Phe	Ala	His	Asp	Ile	Asp	Gln	Lys	Leu	Ser
625					630					635					640
His	Leu	Gly	Ala	Ser	Gln	Leu	Ala	Pro	Thr	Gly	Glu	Gly	Asp	Glu	Leu
				645					650					655	
Ser	Gly	Gln	Glu	Asp	Ala	Phe	Arg	Ser	Trp	Ala	Val	Gln	Thr	Phe	Arg
			660					665					670		
Ala	Ala	Cys	Glu	Thr	Phe	Asp	Val	Arg	Ser	Lys	His	His	Ile	Gln	Ile
		675					680					685			
Pro	Lys	Arg	Phe	Thr	Ser	Asn	Ala	Thr	Trp	Glu	Pro	Gln	Gln	Tyr	Arg
	690					695					700				
Leu	Ile	Gln	Ser	Pro	Glu	Pro	Leu	Asp	Leu	Asn	Arg	Ala	Leu	Ser	Ser
705					710					715					720
Ile	His	Ala	Lys	Asn	Val	Phe	Thr	Met	Arg	Leu	Lys	Ser	Gln	Gln	Asn
				725					730					735	
Leu	Gln	Ser	Glu	Lys	Ser	Ser	Arg	Thr	Thr	Leu	Leu	Val	Gln	Leu	Thr
			740					745					750		
Phe	Glu	Gly	Ser	Arg	Gly	Pro	Ser	Tyr	Leu	Pro	Gly	Glu	His	Leu	Gly
		755					760					765			
Ile	Phe	Pro	Gly	Asn	Gln	Thr	Ala	Leu	Val	Gln	Gly	Ile	Leu	Glu	Arg
	770					775					780				
Val	Val	Asp	Cys	Pro	Thr	Pro	His	Gln	Thr	Val	Cys	Leu	Glu	Val	Leu
785					790					795					800
Asp	Glu	Ser	Gly	Ser	Tyr	Trp	Val	Lys	Asp	Lys	Arg	Leu	Pro	Pro	Cys
				805					810				815		
Ser	Leu	Ser	Gln	Ala	Leu	Thr	Tyr	Phe	Leu	Asp	Ile	Thr	Thr	Pro	Pro
			820					825					830		

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Thr Gln Leu Gln Leu His Lys Leu Ala Arg Phe Ala Thr Asp Glu Thr  
 835 840 845  
 Asp Arg Gln Arg Leu Glu Ala Leu Cys Gln Pro Ser Glu Tyr Asn Asp  
 850 855 860  
 Trp Lys Phe Ser Asn Asn Pro Thr Phe Leu Glu Val Leu Glu Glu Phe  
 865 870 875 880  
 Pro Ser Leu His Val Pro Ala Ala Phe Leu Leu Ser Gln Leu Pro Ile  
 885 890 895  
 Leu Lys Pro Arg Tyr Tyr Ser Ile Ser Ser Ser Gln Asp His Thr Pro  
 900 905 910  
 Ser Glu Val His Leu Thr Val Ala Val Val Thr Tyr Arg Thr Arg Asp  
 915 920 925  
 Gly Gln Gly Pro Leu His His Gly Val Cys Ser Thr Trp Ile Arg Asn  
 930 935 940  
 Leu Lys Pro Gln Asp Pro Val Pro Cys Phe Val Arg Ser Val Ser Gly  
 945 950 955 960  
 Phe Gln Leu Pro Glu Asp Pro Ser Gln Pro Cys Ile Leu Ile Gly Pro  
 965 970 975  
 Gly Thr Gly Ile Ala Pro Phe Arg Ser Phe Trp Gln Gln Arg Leu His  
 980 985 990  
 Asp Ser Gln His Lys Gly Leu Lys Gly Gly Arg Met Ser Leu Val Phe  
 995 1000 1005  
 Gly Cys Arg His Pro Glu Glu Asp His Leu Tyr Gln Glu Glu Met  
 1010 1015 1020  
 Gln Glu Met Val Arg Lys Arg Val Leu Phe Gln Val His Thr Gly  
 1025 1030 1035  
 Tyr Ser Arg Leu Pro Gly Lys Pro Lys Val Tyr Val Gln Asp Ile  
 1040 1045 1050  
 Leu Gln Lys Gln Leu Ala Asn Glu Val Leu Ser Val Leu His Gly  
 1055 1060 1065  
 Glu Gln Gly His Leu Tyr Ile Cys Gly Asp Val Arg Met Ala Arg  
 1070 1075 1080  
 Asp Val Ala Thr Thr Leu Lys Lys Leu Val Ala Thr Lys Leu Asn  
 1085 1090 1095  
 Leu Ser Glu Glu Gln Val Glu Asp Tyr Phe Phe Gln Leu Lys Ser  
 1100 1105 1110  
 Gln Lys Arg Tyr His Glu Asp Ile Phe Gly Ala Val Phe Ser Tyr  
 1115 1120 1125  
 Gly Ala Lys Lys Gly Ser Ala Leu Glu Glu Pro Lys Ala Thr Arg  
 1130 1135 1140

Leu

<210> SEQ ID NO 13  
 <211> LENGTH: 20  
 <212> TYPE: PRT  
 <213> ORGANISM: Mus sp.

&lt;400&gt; SEQUENCE: 13

Gly Asp Pro Ala Asn Val Glu Phe Thr Glu Ile Cys Ile Gln Gln Gly  
 1 5 10 15

Trp Lys Pro Arg  
 20

&lt;210&gt; SEQ ID NO 14

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<211> LENGTH: 20  
 <212> TYPE: PRT  
 <213> ORGANISM: Drosophila sp.

<400> SEQUENCE: 14

Gly Asp Pro Met Asn Val Glu Phe Thr Glu Thr Val Ala Leu Lys Met  
 1                   5                   10                   15  
 Gln Leu Asp Thr  
                   20

<210> SEQ ID NO 15  
 <211> LENGTH: 20  
 <212> TYPE: PRT  
 <213> ORGANISM: Mus sp.

<400> SEQUENCE: 15

Cys Asp Asn Ser Arg Tyr Asn Ile Leu Glu Glu Val Ala Lys Lys Met  
 1                   5                   10                   15  
 Asp Leu Asp Met  
                   20

<210> SEQ ID NO 16  
 <211> LENGTH: 20  
 <212> TYPE: PRT  
 <213> ORGANISM: Mus sp.

<400> SEQUENCE: 16

Gly Asp Pro Ala Asn Val Glu Phe Thr Glu Glu Val Ala Lys Lys Met  
 1                   5                   10                   15  
 Asp Leu Asp Met  
                   20

<210> SEQ ID NO 17  
 <211> LENGTH: 57  
 <212> TYPE: DNA  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic  
 oligonucleotide

<400> SEQUENCE: 17

cgtctagatc tatgactgaa tatgacgtaa tatgacgtaa tggtaccaga tctggcc                   57

<210> SEQ ID NO 18  
 <211> LENGTH: 41  
 <212> TYPE: DNA  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic  
 oligonucleotide

<400> SEQUENCE: 18

aaatgacgta acggaaatga cgtaacggaa atgacgtaac g                   41

<210> SEQ ID NO 19  
 <211> LENGTH: 42  
 <212> TYPE: DNA  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic  
 oligonucleotide

<400> SEQUENCE: 19

aaatgaatta acggaaatga attaacggaa atgaattaac gg                   42

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<210> SEQ ID NO 20
<211> LENGTH: 81
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
        oligonucleotide

<400> SEQUENCE: 20

tgcacgggtt ttcgacgttc actggtagtg tctgatgagg ccgaaaggcc gaaacgcgat      60
gcccataacc accacgctca g                                     81

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<210> SEQ ID NO 21
<211> LENGTH: 100
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
        polynucleotide

<400> SEQUENCE: 21

tcgaccaca gtttcgggtt ttcgagcaag tctgctagtg tctgatgagg ccgaaaggcc      60
gaaacgcgaa gccgtattgc accacgctca tcgagaaggc                               100

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<210> SEQ ID NO 22
<211> LENGTH: 55
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
        oligonucleotide

<400> SEQUENCE: 22

ctagagcttg caagcatgct tgcaagcaag catgcttgca agcatgcttg caagc          55

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<210> SEQ ID NO 23
<211> LENGTH: 34
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
        oligonucleotide

<400> SEQUENCE: 23

ctctagagcg tacgcaagcg tacgcaagcg tacg                                     34

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<210> SEQ ID NO 24
<211> LENGTH: 17
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
        peptide

<400> SEQUENCE: 24

Arg Lys Arg Glu Ile Arg Leu Gln Lys Asn Arg Glu Ala Ala Arg Glu
1           5           10           15

Cys

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<210> SEQ ID NO 25
<211> LENGTH: 4491
<212> TYPE: DNA
<213> ORGANISM: Drosophila sp.

<400> SEQUENCE: 25

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gaattccggt	tttgaaaagt	gaagcaattg	agtgcggccc	gaaaaagaga	gccgcagaaa	60
gtttgcgaac	agaatttaat	caaaaacttg	gagggtaaat	tgtccaagtg	gttcacctgt	120
tggctgcatt	ttaaatcaac	gaggcaaaca	atcagcgcag	aggagctgct	ccacgttccc	180
cggacaagat	gtcgcagcat	ttcacatcga	tatttgagaa	cctgcgattc	gtgaccatca	240
aacgtgcgac	aatgcgcaa	cagcaacagc	agcagcagca	gcaacagcaa	cttcagcagc	300
agcagcagca	gctgcagcaa	cagaaggcac	agacacagca	acaaaatagc	agaaaaatca	360
aaactcaagc	aacgccaacg	ttgaatggca	atgggctctt	gagcggcaat	ccaaatggcg	420
gaggcgggtga	ctcctcgccc	agccatgaag	tggaccatcc	gggtggagca	caaggagctc	480
aagcagcagg	aggcttgcca	tctttaagtg	gcacgccatt	gaggcaccac	aagcgcgcca	540
gtatctccac	agcatcgccct	ccaattcgcg	aacggcgtgg	caccaacacc	agcatcgtgg	600
tcgaactgga	tggcagtggc	agcgggagtg	ggagtggcgg	tgggtggcgt	ggcgttggtc	660
aggggtgcggg	ttgtctccc	tcgggcagct	gcaactgcgtc	cggaaaaagt	tcgcggaac	720
tatcgccgtc	gccgaaaaac	caacagcagc	ccagaaagat	gtcacaggat	tatcggtcgc	780
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tgagactgaa	gaacatcgag	ggcaggccgg	aggtctacga	cacgctgcac	tgcaagggtc	900
gcgagattct	ttcctgctcg	aaggccacct	gtacgagcag	cattatgaac	attggcaatg	960
cggcgggtgga	ggccaggaaa	tccgatctga	tcctcgaaca	cgccaaggac	ttcctcgagc	1020
agtactttac	atcgataaag	cgtacatcat	gtaccgcccc	cgagacgcga	tggaaacagg	1080
tgcgccagag	cattgagacc	actggacact	atcagctaac	cgaaacggag	ctaatttatg	1140
gtgccaaatt	ggcctggcgc	aattcttcac	gttgcatggg	ccgaatacaa	tggtcgaagt	1200
tgcaggtctt	tgactgtcgt	tatgtgacaa	caacaagtgg	catgtttgaa	gccatttgca	1260
atcacattaa	atatgcaaca	aataagggca	acctgagatc	ggccatcacg	atatttccac	1320
aacgcacaga	tgccaagcat	gattatcgca	tttgaataa	ccaattaata	tcttatgccc	1380
gctacaagca	ggcggatgga	aaaatcattg	gcgatcccat	gaatgtggag	tttacagagg	1440
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tctcggccaa	tggtcacgat	cgggactact	ttgattacc	gcccgaattg	atactggaag	1560
ttccgctgac	ccatcccaaa	ttcgaatgg	tctcggatct	gggactgcga	tggtagcccc	1620
tgcccgcggt	atccagtatg	ctgttcgatg	tgggcggcat	tcagtttacg	gccaccacat	1680
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catcgaaact	atttgacgc	gccttatcga	aacgcataaa	ggcaacagtt	ctatatgcca	2220
ccgaaactgg	caaatcggag	cagtatgcga	agcaactttg	tgaactccta	gggcacgcat	2280
tcaatgcaca	gatatattgc	atgtccgact	acgatatatc	ctccattgag	cacgaggcat	2340

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tgtaattgt	tgtggcctcc	acctttggca	acggtgatcc	ccccgaaaac	ggcgagcttt	2400
tctcccagga	attgtatgcg	atgcgtgtcc	aggagtcttc	cgagcatgga	ttgcaggact	2460
ccagcattgg	ctcgtcaaag	tccttcatga	aggccagctc	gcggcaggag	ttcatgaagc	2520
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caagggccac	ggccaggata	cgaatggcct	cccagcccta	aggatagata	ttogaagtaa	4260
tcaaaatagg	agggtgacat	atccaaattc	gagaggaata	ccaagcactt	gctctttttt	4320
ttcttcata	ttcaaatgca	attaatatt	gtcgctttgt	tcattacata	ttcgtatgaa	4380
taacgtttaa	ataaattaca	ttttattatt	gatttcaatg	tacaaatcaa	ttgtgaaatc	4440
aaaatctaaa	tgtaaaata	tatttcaaat	aaacgaatcg	aaaaggaatt	c	4491

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What is claimed is:

1. A method for assessing the effect of a drug on long term memory formation comprising:

- a) administering said drug to an animal having an inducible activator and/or an inducible repressor wherein said inducible activator and/or inducible repressor is/are under the control of a promoter, wherein said activator is a CREB/CREM/ATF-1 subfamily member associated with potentiation of long term memory and said repressor is an antagonist of said activator and is associated with blocking of long term memory;
- b) inducing expression of said activator and/or repressor, wherein inducing expression of said activator and/or repressor comprises activating said promoter effective to induce expression of said activator and/or repressor;
- c) training said animal under conditions appropriate to produce long term memory formation in said animal, wherein conditions appropriate to produce long term memory formation comprise multiple training sessions with a rest interval between training sessions;
- d) assessing long term memory formation in said animal trained in step c); and
- e) comparing long term memory formation assessed in step d) with long term memory formation produced in a control animal to which said drug has not been administered.

2. The method of claim 1 wherein said animal is *Drosophila* and said activator is an activator isoform of dCREB2.

3. The method of claim 1 wherein said animal is a mammal.

4. The method of claim 1 wherein said animal is *Drosophila* and said repressor is a repressor isoform of dCREB2.

5. A method for assessing the effect of a drug on long term memory formation comprising:

- a) administering said drug to an animal;
- b) determining the functional level of activator and/or repressor in said animal relative to the functional level of activator and/or repressor in a control animal to which said drug has not been administered, wherein said activator is a CREB/CREM/ATF-1 subfamily member associated with potentiation of long term memory and said repressor is an antagonist of said activator and is associated with blocking of long term memory;
- c) selecting said animal in step b) having a functional level of activator and/or repressor which differs from the functional level of activator and/or repressor in the control animal to which said drug has not been administered;
- d) training said animal selected in step c) under conditions appropriate to produce long term memory formation in said animal, wherein conditions appropriate to produce long term memory formation comprise multiple training sessions with a rest interval between training sessions;
- e) assessing long term memory formation in said animal trained in step d); and
- f) comparing long term memory formation assessed in step e) with long term memory formation produced in the control animal to which said drug has not been administered.

6. The method of claim 5 wherein said animal is *Drosophila*, said activator is an activator isoform of dCREB2 and said repressor is a repressor isoform of dCREB2.

7. The method of claim 5 wherein said animal is a mammal.

8. The method of claim 5, wherein the functional level of a homodimer of said activator is determined after said drug is administered, relative to the functional level of a homodimer of said activator present in a control animal to which said drug has not been administered.

9. The method of claim 8 wherein said animal is *Drosophila* and said activator is an activator isoform of dCREB2.

10. The method of claim 8 wherein said animal is a mammal.

11. The method of claim 8 further comprising:

- g) selecting said animal having a functional level of homodimer which differs from the functional level of said homodimer present in the control animal to which said drug has not been administered;
- h) training said animal selected in step g) under conditions appropriate to produce long term memory formation in said animal, wherein said conditions appropriate to produce long term memory formation comprise multiple training sessions with a rest interval between training sessions;
- i) assessing long term memory formation in said animal trained in step h); and
- j) comparing long term memory formation assessed in step i) with long term memory formation produced in the control animal to which said drug has not been administered.

12. The method of claim 11 wherein said animal is *Drosophila* and said activator is an activator isoform of dCREB2.

13. The method of claim 11 wherein said animal is a mammal.

14. The method of claim 5, wherein the functional level of a heterodimer of said activator and said repressor is determined after said drug is administered, relative to the functional level of a heterodimer of said activator and said repressor present in a control animal to which said drug has not been administered.

15. The method of claim 14 wherein said animal is *Drosophila*, said activator is an activator isoform of dCREB2 and said repressor is a repressor isoform of dCREB2.

16. The method of claim 14 wherein said animal is a mammal.

17. The method of claim 14 further comprising:

- g) selecting said animal having a functional level of heterodimer which differs from the functional level of said heterodimer present in the control animal to which said drug has not been administered;
- h) training said animal selected in step g) under conditions appropriate to produce long term memory formation in said animal, wherein said conditions appropriate to produce long term memory formation comprise multiple training sessions with a rest interval between training sessions;
- i) assessing long term memory formation in said animal trained in step h); and
- j) comparing long term memory formation assessed in step i) with long term memory formation produced in the control animal to which said drug has not been administered.

18. The method of claim 17 wherein said animal is *Drosophila*, said activator is an activator isoform of dCREB2 and said repressor is a repressor isoform of dCREB2.

19. The method of claim 17 wherein said animal is a mammal.

20. The method of claim 5, wherein the functional level of a homodimer of said repressor is determined after said drug is administered, relative to the functional level of a homodimer of said repressor present in a control animal to which said drug has not been administered.

21. The method of claim 20 wherein said animal is *Drosophila* and said repressor is a repressor isoform of dCREB2.

22. The method of claim 20 wherein said animal is a mammal.

- 23.** The method of claim **20** further comprising:
- g) selecting said animal having a functional level of homodimer which differs from the functional level of said homodimer present in the control animal to which said drug has not been administered;
  - h) training said animal selected in step g) under conditions appropriate to produce long term memory formation in said animal, wherein said conditions appropriate to produce long term memory formation comprise multiple training sessions with a rest interval between training sessions;
  - i) assessing long term memory formation in said animal trained in step h); and
  - j) comparing long term memory formation assessed in step i) with long term memory formation produced in the control animal to which said drug has not been administered.
- 24.** The method of claim **23** wherein said animal is *Drosophila* and said repressor is a repressor isoform of dCREB2.
- 25.** The method of claim **23** wherein said animal is a mammal.
- 26.** A method for screening a pharmaceutical agent for its ability to potentiate or repress formation of long term memory in an animal comprising:
- a) administering said pharmaceutical agent to said animal;
  - b) determining the functional level of activator and/or repressor in said animal relative to the functional level of activator and/or repressor in a control animal to which said pharmaceutical agent has not been administered, wherein said activator is a CREB/CREM/ATF-1 subfamily member associated with potentiation of long term memory and said repressor is an antagonist of said activator and is associated with blocking of long term memory;
  - c) selecting said animal in step b) having a functional level of activator and/or repressor which differs from the functional level of activator and/or repressor in the control animal to which said pharmaceutical agent has not been administered;
  - d) training said animal selected in step c) under conditions appropriate to produce long term memory formation in said animal, wherein conditions appropriate to produce long term memory formation comprise multiple training sessions with a rest interval between training sessions;
  - e) assessing long term memory formation in said animal trained in step d); and
  - f) comparing long term memory formation assessed in step e) with long term memory formation produced in the control animal to which said pharmaceutical agent has not been administered.
- 27.** The method of claim **26** wherein said animal is *Drosophila*, said activator is an activator isoform of dCREB2 and said repressor is a repressor isoform of dCREB2.
- 28.** The method of claim **26** wherein said animal is a mammal.
- 29.** The method of claim **26**, wherein the functional level of a homodimer of activator is determined after said pharmaceutical agent is administered, relative to the functional level of a homodimer of said activator present in a control animal to which said pharmaceutical agent has not been administered.
- 30.** The method of claim **29** wherein said animal is *Drosophila* and said activator is an activator isoform of dCREB2.
- 31.** The method of claim **29** wherein said animal is a mammal.
- 32.** The method of claim **29** further comprising:
- g) selecting said animal having a functional level of homodimer which differs from the functional level of

- said homodimer present in the control animal to which said pharmaceutical agent has not been administered;
  - h) training said animal selected in step g) under conditions appropriate to produce long term memory formation in said animal, wherein conditions appropriate to produce long term memory formation comprise multiple training sessions with a rest interval between training sessions;
  - i) assessing long term memory formation in said animal trained in step h); and
  - j) comparing long term memory formation assessed in step i) with long term memory formation produced in the control animal to which said pharmaceutical agent has not been administered.
- 33.** The method of claim **32** wherein said animal is *Drosophila* and said activator is an activator isoform of dCREB2.
- 34.** The method of claim **32** wherein said animal is a mammal.
- 35.** The method of claim **26**, wherein the functional level of a heterodimer of said activator and said repressor is determined after said pharmaceutical agent is administered, relative to the functional level of a heterodimer of said activator and said repressor present in a control animal to which said pharmaceutical agent has not been administered.
- 36.** The method of claim **35** wherein said animal is *Drosophila*, said activator is an activator isoform of dCREB2 and said repressor is a repressor isoform of dCREB2.
- 37.** The method of claim **35** wherein said animal is a mammal.
- 38.** The method of claim **35** further comprising:
- g) selecting said animal having a functional level of heterodimer which differs from the functional level of said heterodimer present in the control animal prior to which said pharmaceutical agent has not been administered;
  - h) training said animal selected in step g) under conditions appropriate to produce long term memory formation in said animal, wherein conditions appropriate to produce long term memory formation comprise multiple training sessions with a rest interval between training sessions;
  - i) assessing long term memory formation in said animal trained in step h); and
  - j) comparing long term memory formation assessed in step i) with long term memory formation produced in the control animal to which said pharmaceutical agent has not been administered.
- 39.** The method of claim **38** wherein said animal is *Drosophila*, said activator is an activator isoform of dCREB2 and said repressor is a repressor isoform of dCREB2.
- 40.** The method of claim **38** wherein said animal is a mammal.
- 41.** The method of claim **26**, wherein the functional level of a homodimer of said repressor is determined after said pharmaceutical agent is administered relative to the functional level of a homodimer of said repressor present in a control animal to which said pharmaceutical agent has not been administered.
- 42.** The method of claim **41** wherein said animal is *Drosophila* and said repressor is a repressor isoform of dCREB2.
- 43.** The method of claim **41** wherein said animal is a mammal.
- 44.** The method of claim **41** further comprising:
- g) selecting said animal having a functional level of homodimer which differs from the functional level of said homodimer present in the control animal to which said pharmaceutical agent has not been administered;
  - h) training said animal selected in step g) under conditions appropriate to produce long term memory formation in said animal, wherein conditions appropriate to produce

long term memory formation comprise multiple training sessions with a rest interval between training sessions;

i) assessing long term memory formation in said animal trained in step h); and

j) comparing long term memory formation assessed in step i) with long term memory formation produced in the control animal to which said pharmaceutical agent has not been administered.

45. The method of claim 44 wherein said animal is *Drosophila* and said repressor is a repressor isoform of dCREB2.

46. The method of claim 44 wherein said animal is a mammal.

47. A method for screening a pharmaceutical agent for its ability to modulate long term memory in an animal comprising:

a) administering said pharmaceutical agent to an animal having an inducible activator or an inducible repressor, wherein said activator is a CREB/CREM/ATF-1 subfamily member associated with potentiation of long term memory and wherein said repressor is an antagonist of said activator and is associated with blocking of long term memory;

b) inducing expression of said activator or said repressor;

c) training said animal under conditions appropriate to produce long term memory formation in said animal, wherein conditions appropriate to produce long term memory formation comprise multiple training sessions with a rest interval between training sessions;

d) assessing long term memory formation in said animal trained in step c); and

e) comparing long term memory formation assessed in step d) with long term memory formation produced in a control animal to which said pharmaceutical agent has not been administered.

48. The method of claim 47 wherein said animal is *Drosophila*, said repressor is a repressor isoform of dCREB2 and said activator is an activator isoform of dCREB2.

49. The method of claim 47 wherein said animal is a mammal.

50. A method for assessing the effect of a drug on long term memory formation comprising:

a) administering said drug to *Drosophila* having an inducible repressor isoform of dCREB2 associated with long term memory or an inducible activator isoform of dCREB2 associated with long term memory;

b) inducing expression of said repressor isoform or said activator isoform;

c) subjecting the *Drosophila* to classical conditioning and to at least one odorant and electrical shock; and

d) assessing the performance index of said classical conditioning, wherein said performance index comprises the normalized average of the percent of correct responses, wherein said normalization is effected so that a performance index of zero results from an average of correct responses that is equal to chance, and a performance index of 1 results from an average of correct responses that corresponds to 100% correct responses, and wherein the effect of said drug is assessed by comparing said performance index in the presence of drug to the performance index obtained by subjecting a *Drosophila* to classical conditioning and to at least one odorant and electrical shock in the absence of said drug.

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